Chapter 3

Results
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The cellular functions of the ORF3 protein of HEV remain obscure. The first step towards the identification of the role of ORF3 in infected hepatocytes was to find out host proteins that interact specifically with ORF3. The yeast two-hybrid system is a powerful technique for identifying proteins that interact with a specific protein of interest. In this study we have used the yeast two-hybrid system to screen a healthy human cDNA liver library to identify ORF3 protein’s cellular interacting partners.

The results are presented in two sections. Section 3.1 details the cDNA liver library amplification and screening procedures. Yeast two-hybrid screening with ORF3 as bait revealed several putative ORF3-binding proteins. The different clones were sequenced and the open reading frame was checked. The clones expressing proteins in the correct reading frame were selected. Initial studies showed hemopexin and fibrinogen Bβ, among those selected, to be most relevant for further analysis. The interaction between ORF3-hemopexin and ORF3-fibrinogen Bβ was independently verified by standard biochemical assays including in vitro pull-downs and co-immunoprecipitations in mammalian cells. Co-localization and FRET were used to show in vivo interaction. The ORF3 interaction domain was also mapped. During the investigation it was observed that the total secreted fibrinogen from ORF3 expressing hepatocytes was less compared to vector transfected cells. Since fibrinogen’s role as a positive regulator of the innate immune response is well documented, we took keen interest in its interaction with ORF3. Section 3.2 details the studies conducted to explore the mechanism(s) of ORF3 dependent decrease in secreted fibrinogen.
Aim: To identify cellular interacting partners of the ORF3 protein of Hepatitis E virus from human liver cDNA library by yeast two-hybrid system

3.1.1. Screening for cellular proteins that interact with the ORF3 protein of HEV

To identify host proteins that interact with the ORF3 protein of HEV, ORF3 was employed in Matchmaker (Clontech) GAL4-based yeast two-hybrid screen of a healthy human liver GAL4 AD fusion cDNA library. The ORF3 protein expressed as a fusion to the GAL4 DNA binding domain (DNA-BD) of pAS2 (Tyagi et al., 2004) was used as bait. A human liver cDNA AD fusion library constructed in AD vector, pACT2, such that the proteins encoded by the inserts are fused to the GAL4 AD, was obtained as E. coli transformants from Clontech. Figure 3.1.1 provides an overview of strategies employed to screen for cellular proteins that interact with HEV ORF3 protein.

3.1.1.1. Library titering and amplification

The library obtained had one million independent clones. The library was first amplified to produce enough plasmid DNA to screen the library in yeast. About 100-500 μg of plasmid DNA is required to screen around one million independent clones. In order to appropriately amplify the library, 15 μl of the original library was required to be plated, determined as follows:

\[
\text{Amount of library to be plated (μl)} = \frac{\text{Number of clones to be screened}}{\text{Library titer (CFU/ml)}}
\]

where: number of clones to be screened was \(3 \times 10^6\) and library titer, as determined by the dilution method, was found to be \(2 \times 10^8\) CFU/ml.

Plating at the density of 10,000 CFU/plate, the exact number of plates required to screen \(3 \times 10^6\) clones was calculated to be 300.

\[
\text{Amount of plates required} = \frac{\text{Number of clones to be screened}}{\text{CFU per plate}}
\]
Fig. 3.1.1. Using a GAL4-based two-hybrid system to screen an AD fusion library for proteins that interact with a bait protein.

After doing the suitable calculations, finally, 15 µl of the original library was appropriately diluted and spread on 300 LB agar plates containing ampicillin. The plates were incubated at 37°C for 18-24 hr and plasmid was prepared from the pooled bacterial suspension as described under materials and methods.
3.1.1.2. Library transformation and screening

Yeast host strain AH109 was transformed with pAS2-ORF3 and then sequentially transformed with the AD fusion library (pACT2-LL). The co-transformants were selected for growth on minimal synthetic dropout (SD) medium [comprised of a nitrogen base, a carbon source (glucose) and amino acid dropout supplement] lacking leucine and tryptophan (SD-leu’trp’) to select pACT2-LL and pAS2-ORF3, respectively.

The transformation efficiency and number of clones screened were determined. To calculate transformation efficiency (CFU/μg) the number of colony forming units (CFU) obtained on the SD-leu’trp’ dilution plate that had 30-300 CFU, were counted. About 200 μg of amplified plasmid DNA was resuspended in 30 ml LB. 100 μl of a 1:100 dilution of this suspension produced 70 colonies. The transformation efficiency was found to be 1.05 X 10⁴ using formula:

\[
\text{Transformation efficiency (CFU/µg DNA)} = \frac{\text{CFU} \times \text{Total suspension (µl)}}{\text{Vol. plated (µl)) \times \text{Dilution factor} \times \text{Amt. of DNA used (µg)}}
\]

where: CFU was 70; total suspension was 30,000 µl; 100 µl volume was plated at dilution 0.01 and amount of DNA used was 200 µg.

The number of clones finally screened were \(2 \times 10^6\), determined by formula:

\[
\text{Number of clones screened} = \frac{\text{Transformation efficiency (CFU/µg) \times Amt. of library plasmid used (µg)}}{\text{Transformation efficiency (CFU/µg) \times Amt. of library plasmid used (µg)}}
\]

The co-transformants that contain library fusion proteins, which interact with each other, activate transcription of three reporter genes: histidine, adenine and lac Z gene. After about 3 days of incubation at 30°C, the co-transformants that were selected on SD-leu’trp’ were tested for histidine (His⁺, clones expressing the HIS3 reporter gene; i.e., they do not require His in the medium to grow) and adenine (Ade⁺, clones expressing the ADE2 reporter gene; i.e., they do not require Ade in the medium to grow) prototrophy, by first plating on triple negative synthetic dropout low stringency media, SD-leu’trp’his⁺. Grown colonies were further selected on quadruple negative high stringency media SD-leu’trp’his⁺ade⁺. The His⁺Ade⁺ prototrophs were further tested for LacZ⁺ phenotype (clones expressing the LacZ reporter gene; i.e., they are positive for β-galactosidase activity) by colony -lift filter assay. Finally, from \(2 \times 10^6\) independent library clones screened, 569 colonies were obtained that were His⁺Ade⁺LacZ⁺ and harbored putative ORF3 interacting proteins. Original colonies...
were collected within grids or orderly arrays on master plates. To further analyze these His' Ade' LacZ' clones, the strategy schematically shown in Fig. 3.1.2, was followed.

**Fig. 3.1.2. A detailed overview of strategies for analyzing and verifying putative positive clones obtained from the yeast two-hybrid screen.**
3.1.1.3. Isolation and sorting of plasmid DNA from putative positive yeast clones

Out of the 569 colonies obtained, 200 co-transformants were selected at random. The His\(^+\) Ade\(^+\) LacZ\(^-\) phenotype was reconfirmed by streaking the colonies on SD-leu\(\text{trp}\) his\(\text{ade}\) medium and assaying for \(\beta\)-gal activity again. The plasmid DNA was isolated from these positive colonies by the lyticase method. Even after persistent trials, no AD/library plasmids were obtained from 43 yeast clones; hence these were not pursued further. The AD/library plasmids were isolated from the remaining 157 clones. Since the plasmid DNA isolated from each yeast colony is a mixture of the DNA-BD/bait and AD/library plasmid, the latter were rescued via transformation of E. coli strain HB101 and plating on M9 medium lacking leucine. HB101 cells have a defect in leuB that can be complemented by yeast LEU2. Thus only those bacterial cells transformed with the AD/library plasmid grow on M9 medium lacking leucine, while those harboring DNA-BD/bait plasmid or the untransformed cells fail to grow.

The AD/library plasmids were reisolated from bacteria and sorted to eliminate duplicates. This was done by subjecting the plasmids to HindIII and BgII restriction enzyme digestions separately. The HindIII digestion releases the cDNA insert along with the fused AD (~750 bp in size) and BgII digestion releases the cloned cDNA insert by cutting at sites flanking the multiple cloning site of pACT2 vector (Refer to pACT2 plasmid map, Fig. 2.1, pg. 53). The fragments generated by restriction enzyme digestion were analyzed by agarose gel electrophoresis. The 157 AD/library plasmids analyzed fall within primarily 27 distinct groups, each member of a group giving the same sized fall-outs when digested with HindIII or BgII. A new master plate was prepared with two representative clones from each group and the other duplicates were discarded. The restriction enzyme digestion pattern of a single representative from each group, digested with HindIII (Fig. 3.1.3A and B) and BgII (Fig. 3.1.4A and B) is shown.

The AD/library inserts from each group were also PCR amplified using plasmid DNA isolated from yeast as template and primers ADAFP and ADARP. Amplified PCR products of different sizes were obtained, each member of a group giving single band of the same size, again showing that each member of a group harbors the same AD/library plasmid. Amplified fragments of a single representative member from each group are shown (Fig. 3.1.5A and B).
**Fig. 3.1.3. HindIII restriction digestion analysis.** Fragments obtained by digesting a representative clone from each group (A, top, group 1-19 and B, left, group 20-27) with HindIII were run on 1% agarose gel. 

*Fig. 3.1.4. BglII restriction digestion analysis.** Fragments obtained by digesting a representative clone from each group (A, top, group 1-19 and B, left, group 20-27) with BglII were run on 1% agarose gel. 

*M is the molecular weight marker in base pairs. The position of each band of marker is numbered to the right of the panels. (1, 21226; 2, 5148; 3, 4973; 4, 4268; 5, 3230; 6, 2027; 7, 1904; 8, 1584; 9, 1375; 10, 947; 11, 831; 12, 564).*
Fig. 3.1.5. **PCR amplification of cDNA inserts.** PCR amplified products of representative clone from each group (A, group 1-19 and B, group 20-27) were run on 1% agarose gel. M is the molecular weight marker in base pairs. The position of each band of marker is numbered to the right of the panels. (1, 21226; 2, 5148; 3, 4973; 4, 4268; 5, 3230; 6, 2027; 7, 1904; 8, 1584; 9, 1375; 10, 947; 11, 831; 12, 564).

### 3.1.1.4. Sequence analysis of the cDNA inserts of isolated clones

The cDNA inserts in the AD/library plasmids, from one or two clones in each group were sequenced using the 5' AD sequencing primer, ADSFP, to identify the interacting partner. Sequence comparisons were done by FASTA searches of the GenBank database. After sequencing the clone and BLAST analysis (http://www.ncbi.nlm.nih.gov/), the different cDNA inserts were identified. The ‘Basic Local Alignment Search Tool’ (BLAST) finds regions of local similarity between sequences. The program compares input nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. The value is given as Expect value (E), where E of 0.0 indicates the match is significant. Percent identity signifies the percentage of nucleotides that exactly match between the submitted ‘query’ and GenBank database ‘subject’ sequence. The BLAST results are tabulated in Table 3.1.1.
Table 3.1.1. BLAST results

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>No. of clones</th>
<th>Identity (%)</th>
<th>Expect value</th>
<th>GenBank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mRNA for alpha 1 microglobulin and bikunin precursor</td>
<td>11</td>
<td>98</td>
<td>1e-109</td>
<td>NM_001633</td>
</tr>
<tr>
<td>2</td>
<td>mRNA for alpha 1 microglobulin and bikunin precursor</td>
<td>13</td>
<td>99</td>
<td>1e-109</td>
<td>NM_001633</td>
</tr>
<tr>
<td>3</td>
<td>Fibrinogen beta chain (FGB) mRNA</td>
<td>19</td>
<td>100</td>
<td>0.0</td>
<td>NM_005141</td>
</tr>
<tr>
<td>4</td>
<td>BAC clone RP11-701P16</td>
<td>1</td>
<td>93</td>
<td>2e-133</td>
<td>AC084871</td>
</tr>
<tr>
<td>5</td>
<td>Hemopexin (Hpx) mRNA</td>
<td>5</td>
<td>98</td>
<td>0.0</td>
<td>NM_000613</td>
</tr>
<tr>
<td>6</td>
<td>Fibrinogen beta chain (FGB) mRNA</td>
<td>5</td>
<td>100</td>
<td>0.0</td>
<td>NM_005141</td>
</tr>
<tr>
<td>7</td>
<td>STAG-3-like mRNA</td>
<td>3</td>
<td>100</td>
<td>0.0</td>
<td>NM_001025202</td>
</tr>
<tr>
<td>8</td>
<td>Fibrinogen beta chain (FGB) mRNA</td>
<td>2</td>
<td>100</td>
<td>0.0</td>
<td>NM_005141</td>
</tr>
<tr>
<td>9</td>
<td>START domain containing 5 (STARD5), transcript variant 1, mRNA</td>
<td>3</td>
<td>100</td>
<td>0.0</td>
<td>NM_1819002</td>
</tr>
<tr>
<td>10</td>
<td>Chondroitin polymerizing factor (CHPF) mRNA</td>
<td>11</td>
<td>99</td>
<td>0.0</td>
<td>NM_024536</td>
</tr>
<tr>
<td>11</td>
<td>Protein phosphatase 6, catalytic subunit (PPP6C) mRNA</td>
<td>12</td>
<td>100</td>
<td>1e-72</td>
<td>NM_002721</td>
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<tr>
<td>12</td>
<td>RAP1B, member of RAS oncogene family (RAP1B), transcript variant 1 mRNA</td>
<td>7</td>
<td>98</td>
<td>1e-150</td>
<td>NM_015646</td>
</tr>
<tr>
<td>13</td>
<td>X-ray repair complementing defective repair in Chinese hamster cells 3 (XRCC3) gene, complete cds</td>
<td>6</td>
<td>99</td>
<td>0.0</td>
<td>AF508041</td>
</tr>
<tr>
<td>14</td>
<td>Eukaryotic translation initiation factor 5 (EIF5), transcript variant 1, mRNA</td>
<td>8</td>
<td>99</td>
<td>0.0</td>
<td>NM_001969</td>
</tr>
<tr>
<td>15</td>
<td>mRNA for FLJ00221 protein</td>
<td>2</td>
<td>99</td>
<td>0.0</td>
<td>AK074148</td>
</tr>
<tr>
<td>16</td>
<td>Sulfatase modifying factor 2 (SUMF2), transcript variant 3, mRNA</td>
<td>4</td>
<td>99</td>
<td>0.0</td>
<td>NM_001042469</td>
</tr>
<tr>
<td>17</td>
<td>Glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding), mRNA</td>
<td>6</td>
<td>100</td>
<td>0.0</td>
<td>BC084553</td>
</tr>
<tr>
<td>18</td>
<td>Ferritin, light polypeptide, mRNA</td>
<td>15</td>
<td>99</td>
<td>0.0</td>
<td>NM_000146</td>
</tr>
</tbody>
</table>
The results of the BLAST search identified 27 different clones in the GenBank database. From the sequences, the presence of an open reading frame fused to the GAL4 AD sequence was verified. The results indicated that clones from several groups contained ORFs in the incorrect frame to the GAL4 AD coding sequence (groups 7, 10, 18, 19, 21, 24 and 26). A few encoded for novel genes whose cDNA was found to have no strong homology to any other previously characterized sequence (groups 4, 11, 20, 23 and 27). The clone in group 15 encoded for a hypothetical protein, FLJ00221. Two clones contained cDNA inserts in the reverse orientation (groups 13 and 25). Clones in groups 12 and 17 contained only non-translated sequences. In group 22, only a very short sequence matched with the protein, TBXA2R.

One question that now comes forward is that how such clones showed up as ORF3-associated proteins? Some positive clones were obtained with in-frame stop codons. In some cases in-frame stop codons make the ORF after the GAL4 AD only code for a very short peptide. The short peptide, when fused to the GAL4 AD may cause a significant structural change of the GAL4 AD or create a new epitope that now becomes a bait-specific interacting fusion protein. Therefore it is true positive by
the standard of the yeast two-hybrid system; but it is also a false negative in a biological sense. Nontranslated gaps upstream of ORF inserts can occur in cDNA libraries due to the cloning of a portion of the 5' untranslated region of the mRNA along with the coding region in the cDNA. Further a large ORF in the wrong reading frame may actually correspond to the expressed protein as yeast may actually be able to do a frame switch (translational read through). Fromont-Racine et al. (1997) in large-scale analysis of a functional complex by two-hybrid methods, isolated several inserts with out-of-frame fusions. For some of these, the authors showed independently that the fusions produced functional proteins. Therefore, at least under the conditions chosen, translational frameshifting seems to be a significant mechanism of protein expression in yeast. A cDNA in reverse orientation could actually be transcribed from a cryptic promoter with the ADH1 terminator in pACT2. Although there is no experiment or literature-based evidence for this, Chien et al. (1991) reported a cryptic promoter present in the ADH terminator region. In our judgment, however, all such interactions that were isolated from the library screen were not significant and these types of isolates were not pursued further.

Out of the 27 groups, cDNA inserts from 9 groups (groups 1, 2, 3, 5, 6, 8, 9, 14 and 16) encoded in frame AD fusion proteins including alpha 1 microglobulin bikunin precursor, fibrinogen Bβ, hemopexin, STARD5, EIF5 and SUMF2. Proteins encoded by these select groups and their functions are briefly discussed below.

**Group 1 and 2:** α1 microglobulin bikunin precursor (AMBP) is a known ORF3 interacting protein (Tyagi et al., 2004). The members of both these groups coded for this protein but with different extents of N-terminal deletions. AMBP codes for two unrelated plasma glycoproteins, α1 microglobulin and bikunin. α1 microglobulin is a member of the lipocalin superfamily and is postulated to have a role in immune regulation (Akerstrom, 1992). Bikunin is a kunitz-type serine protease inhibitor (Bratt et al., 1994). Both these proteins also interact with ORF3 (Tyagi et al., 2004, 2005).

**Group 9:** The steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain is a protein module of ~210 residues that binds lipids, including sterols (Ponting and Aravind, 1999). Fifteen mammalian proteins, STARD1-STARD15, possess a START domain. The START domain can be regarded as a lipid-exchange and/or a lipid sensing domain. Mammalian START proteins have diverse expression patterns and can be found free in the cytoplasm, attached to membranes or in the nucleus. They appear to function in a variety of distinct
physiological processes, such as lipid transfer between intracellular compartments, lipid metabolism and modulation of signaling events (Alpy and Tomasetto, 2005; Rodriguez-Agudo et al., 2005).

**Group 14:** Eukaryotic translation initiation factor 5 (EIF5) interacts with the 40S initiation complex to promote hydrolysis of bound GTP with concomitant joining of the 60S ribosomal subunit to the 40S initiation complex. The resulting functional 80S ribosomal initiation complex is then active in peptidyl transfer and chain elongations (Das et al., 1997).

**Group 16:** The sulfatases comprise a large family of prokaryotic and eukaryotic enzymes that catalyze the hydrolysis of ester sulfates (Hopwood & Ballabio, 2001). Sulfatases undergo a unique post-translational modification that converts a highly conserved cysteine located within their active site into formylglycine. This modification is necessary for the catalytic activities of the sulfatases, and it is generated by the protein product of sulfatase-modifying factor 1 (SUMF1) and 2 (SUMF2) (Schmidt et al., 1995; Zito et al., 2005).

STARD5, EIF5 and SUMF2 genes are not exclusively expressed in the liver. These proteins are involved in general housekeeping activities within the cell. The ORF3 protein’s interaction with these proteins definitely shows ORF3 is involved in various cellular functions. In fact, ORF3 protein’s probable association with STARD5 further strengthens the previous observations that ORF3 is involved in cellular signal transduction pathways. STARD5, EIF5 and SUMF2 were not pursued further.

Of particular interest to us were the group 3, 6 and 8 coding for fibrinogen Bβ and group 5 coding for hemopexin.

**Group 3, 6 and 8:** Fibrinogen is an acute phase plasma glycoprotein synthesized constitutively by liver epithelium, the synthesis of which is upregulated 2-10 fold following infections, tissue injury and inflammation (Grieninger et al., 1983). It is a large, 340 kDa dimeric molecule, each unit of which is composed of three non-identical subunit proteins; A alpha (Aα, M r 66000), B beta (Bβ, M r 52000) and gamma (γ, M r 46500) (Redman and Xia, 2001). Apart from its well established role in hemostasis, fibrinogen has been proposed to be one of the key regulators of the innate immune response.

**Group 5:** The hemopexin (Hpx) protein, mainly produced by the parenchymal cells of the liver, is an acute phase protein (Tolosano and Altruda, 2002). It is the major
heme transporter in plasma, is a player in iron homeostasis and also regulates gene expression (Alam and Smith, 1989, 1992) to promote cell survival. These activities of Hpx become significant during acute infection (Delanghe and Langlois, 2001). Hpx domains are found as C-terminal domains in matrix metalloproteinases (MMPs). MMPs are Zn$^{2+}$- and Ca$^{2+}$- dependent endopeptidases, which function in the turn over of ECM components, and in the normal immune response to infections (Das et al., 2003). MMPs have diverse roles: facilitate leukocyte recruitment, cytokine and chemokine processing, defensin activation, i.e.; they modulate the immune response to infection and also are involved in matrix remodeling (McCawley and Matrisian, 2001; Parks et al., 2004).

Previous studies in the lab have shown that ORF3 protein promotes secretion of $\alpha_1$ microglobulin, whose inhibitory effects on the immune system are well documented (Akerstrom, 1992), suggesting that it creates an immunosuppressed environment around the infected hepatocytes. The ORF2 protein of HEV was also shown to induce endoplasmic reticulum stress leading to lowering of NF$\kappa$B activity and an immunosuppressed microenvironment (Surjit, 2005). This implies that perhaps during the course of natural infection both the proteins of HEV play a role in creating an immunosuppressed milieu around the infected cells. Because of the involvement of both hemopexin and fibrinogen in the normal immune response to infections, they were of considerable interest to us. Their interaction with ORF3 may interfere with their role as acute phase reactants. Thus fibrinogen $\beta$ and hemopexin proteins were selected for further analysis.

3.1.1.5. Detailed analysis of pACT2-Hpx and pACT2-$\beta$ cDNA clones

Fig. 3.1.6 shows the $Bgl$II and $Hind$III digestion profile of pACT2-$\beta$ and pACT2-Hpx. The human hemopexin (Hpx) mRNA (GenBank reference sequence NM_000613), was independently found 5 times from the library screening. The cDNA was ~1.6 kb, as obtained by $Bgl$II digestion (Fig. 3.1, lane 3) and coded for the full-length Hpx protein. $Hind$III digestion gave fallout of a ~2.3 kb band (lane 4). Finally, we continued our studies on this clone from group 5. Human fibrinogen $\beta$ mRNA (GenBank reference sequence NM_005141), was independently found many times from the library screen. The ~1.45 kb cDNA, as obtained by $Bgl$II digestion (lane 1), was isolated from 19 different clones (group 3) from the library screen.
HindIII digestion gave fallout of ~2.1 kb (lane 2). Finally, we continued our studies on this clone from group 3.

![Liberace](image)

**Fig. 3.1.6. BglII and HindIII restriction enzyme analysis.** pACT2-Bβ and pACT2-Hpx library isolates were digested with BglII (lanes 1 and 3, respectively) and HindIII (lanes 2 and 4, respectively). The digested samples were run on 1% agarose gel. M is the molecular weight marker in base pairs. The position of each band of marker is numbered to the left of the panel. (1, 21226; 2, 5148; 3, 4973; 4, 4268; 5, 3230; 6, 2027; 7, 1904; 8, 1584; 9, 1375; 10, 947; 11, 831; 12, 564).

The pACT2-Bβ clone, from group 3, contained an ORF which encoded N-terminally truncated fibrinogen Bβ protein lacking the first 18 amino acids of the coding sequence. The clones in group 6 (5 clones in total) and 8 (two clones) also encoded for N-terminally truncated fibrinogen Bβ. Group 6 encoded for 323 amino acids N-terminally truncated protein, while group 8 lacked N-terminal 381 amino acids.

The selected fibrinogen Bβ and Hpx clones, were also sequenced from the 3' end using 3' AD sequencing primer ADSRP, to confirm whether the C-terminal coding sequence was intact. Indeed, the fibrinogen Bβ clone lacked only the N-terminal 18 amino acids and the Hpx clone coded for the full-length protein. The blast results for these two clones when sequenced from 5' end are given in Fig. 3.1.7A and B and 3.1.8A and B.
Fig. 3.1.7A. The query sequence of the pACT2-Bf clone that was put in the BLAST search engine is given (5' to 3'). The sequence in green codes for the amino acids 873-881 of the GAL4 AD, the sequence in black is the pACT2 vector encoded sequence, while the sequence in pink shows the linker peptide used for cloning the cDNA. The fibrinogen Bf sequence is shown in blue. The first codon in its sequence coding for amino acid 19 is shown in capitals. The amino acids encoded are indicated below the sequence.
Fig. 3.1.7B. BLAST result of a representative clone from group 3 encoding N-terminal truncated fibrinogen Bβ.
**Fig. 3.1.8A.** The query sequence of the pACT2-Hpx clone that was put in the BLAST search engine is given (5' to 3'). The sequence in green codes for the amino acids 873-881 of the GAL4 AD, the sequence in black is the pACT2 vector encoded sequence, while the sequence in pink shows the linker peptide used for cloning the cDNA. The Hpx sequence is shown in blue. The first codon in its sequence coding for the first amino acid methionine is shown in capitals. The amino acids encoded are indicated below the sequence.
Since all combinations of protein-protein interactions are assayed, the possibility of identifying artifactual partners exists and is a typical disadvantage of all exhaustive screening procedures. Due to the so-called time/space constraints, it is potentially possible that both proteins, although able to interact, are never in close proximity to each other within the cell. So once two interacting partners are identified, the biological relevance of the interaction remains to be determined. Consequently, ORF3-Hpx and ORF3-Bβ interactions were analyzed and verified to be 'true'
interactions in the yeast two-hybrid sense and further verified in mammalian cells to show the interactions are physiologically relevant.

A detailed overview of strategies for analyzing and verifying putative positive clones obtained from the yeast two-hybrid screen has been outlined in Fig. 3.1.2. For a two-hybrid interaction obtained from a screen it is necessary to first verify that the activation of the reporter genes in His'Ade'LacZ' transformants requires the interaction of both the hybrid proteins i.e., DNA-BD/bait and AD/library plasmid to eliminate false positives. Two classes of false positives are normally encountered in a library screen. The first are those AD/library plasmids, which can turn on reporter gene activity by themselves in absence of the DNA-BD/bait. The other class is those AD/library plasmids, which also turn on reporter gene activity, however, require the empty DNA/BD plasmid for this activity (Bartel et al., 1993). A true positive colony exhibits reporter gene expression only when it contains the DNA-BD/bait plasmid and an AD/library plasmid that encode fusion proteins that interact with each other. Thus as a first step, interaction in yeast must be confirmed by co-transforming DNA-BD/bait or DNA-BD vector and AD/library plasmids into AH109. Further, to retest the ability of the two-hybrid proteins to interact specifically, additional two-hybrid transformations must be done to confirm the interaction. We used yeast mating (Harper et al., 1993; Finley and Brent, 1994) for this purpose. An obvious critique concerns the extensive use of chimeras. The fusion might change the actual conformation of the bait and/or prey and consequently alter functionalities. The reciprocal transfer of proteins, i.e. switching proteins from DNA-binding fusions to activating domain fusions and vice versa, alternatively called ‘domain swapping’ experiment, provides confirmation that the interaction is independent of the fused domains (Chien et al., 1991; van Aelst et al., 1993). To obtain data on the relative strength of the two-hybrid interaction, quantitative β-galactosidase assays were performed using CPRG as substrate. The interaction was also assayed in presence of different mM concentrations of 3-AT, to obtain strength of interaction as a function of histidine prototrophy. The interactions then were confirmed using other standard biochemical techniques. In the following two subsections, 3.1.2 and 3.1.3, the results of the analysis and verification of ORF3-Hpx and ORF3-Bβ interaction are detailed, respectively.
3.1.2. Analysis and verification of ORF3-Hpx interaction

3.1.2.1. Yeast two-hybrid analysis

In order to confirm the ORF3-Hpx interaction, the Hpx AD/library plasmid was reintroduced into AH109 along with BD-ORF3 (Fig. 3.1.9).

<table>
<thead>
<tr>
<th>Two-hybrid transformants</th>
<th>Leu' Trp'</th>
<th>Leu' Trp' His'</th>
<th>Leu' Trp' His' Ade'</th>
<th>50 mM 3-AT</th>
<th>Filter β-gal</th>
<th>Relative liquid β-galactosidase units</th>
</tr>
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<tbody>
<tr>
<td>AH109</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BD-</td>
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</tr>
<tr>
<td>AD-</td>
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As a positive control for the assay, self-association of ORF3 that has been reported earlier (Tyagi et al., 2001) was observed. The empty BD- and AD- vectors and bait (BD-ORF3) and prey (AD-Hpx) alone or in combination (BD- + AD- and BD- + AD-Hpx), served as negative controls. Our results showed that the histidine, adenine and β-galactosidase reporter activity was specifically turned on only when ORF3 and Hpx were present together inside the yeast host, allowing growth on
synthetic dropout medium lacking leucine, tryptophan, histidine and adenine (leu’trp’his’ade’) and blue color on the filter β-galactosidase assay. A quantitative estimation of the relative strengths of interaction between ORF3 and Hpx in comparison to controls as judged by liquid β-galactosidase assay is also shown graphically in Fig. 3.1.9. The liquid β-galactosidase levels were almost 30 fold higher compared to the negative controls and about 1.3 fold of the positive control. The histidine reporter construct has residual histidine expression that is overcome by growing cells in the presence of 3-amino 1, 2, 4 triazole (3-AT). Only the ORF3 and Hpx co-transformants and the positive control were able to grow in the presence of 50 mM 3-AT, clearly displaying specificity and strength of the interaction.

The interaction was also verified using the yeast mating protocol. The AH109 stain (mating type a) was transformed with BD-ORF3 and Y187 (mating type α) with AD-Hpx. The two strains were mated and diploids were analyzed for reporter gene activity. All appropriate positive and negative controls were used as above. The results (Fig. 3.1.10) clearly showed that ORF3 and Hpx interact with each other. Additionally, when Hpx was switched from AD- to the DNA-BD vector and ORF3 from the BD- to the AD- vector (domain swapping), a positive two-hybrid interaction was again obtained, showing specificity of the interaction. To further validate our findings, Hpx full-length cDNA was independently cloned from human hepatoma cells (HuH-7), in the yeast two-hybrid AD- vector, pGADT7. Hpx expression from this construct, pGADT7-Hpx (HuH-7) was checked by coupled in vitro transcription-translation. Translated lysate was analyzed by SDS-PAGE followed by autoradiography. Fig. 3.1.11 shows the expressed Hpx protein of the expected size of 53 kDa. When AD-Hpx (HuH-7) was co-transformed with BD-ORF3, a positive yeast two-hybrid interaction was observed (Fig. 3.1.10).

3.1.2.2. In vitro analysis

The yeast two-hybrid analysis clearly demonstrated that Hpx could turn on reporter gene activity only when ORF3 was co-expressed in the same cell. The next immediate challenge was to confirm this interaction outside the yeast two-hybrid system.
Fig. 3.1.10. Results of yeast two-hybrid retest with Hpx. AH109a (mating type a) and Y187a (mating type a) are two haploid host cells transformed with either BD- or AD- plasmids or fused BD- and AD- expression constructs of ORF3 and Hpx. Mating the two produce diploid cells (separated by slash, /), the co-transformants containing ORF3 and Hpx together, turn on reporter gene activity. The abbreviations used and negative and positive controls are as described in Fig. 3.1.9. A positive two-hybrid interaction was also obtained when the Hpx protein was expressed as a fusion to the BD- vector instead of to AD- and ORF3 vice versa (domain swapping). AD-Hpx (HuH-7) represents Hpx cDNA independently isolated from human hepatoma cells (HuH-7) and cloned as a fusion to AD-. As does the library isolate, this clone also gave a positive two-hybrid interaction with ORF3.

Fig. 3.1.11. In vitro expression of Hpx (HuH-7) Hpx protein expression was verified by in vitro coupled transcription-translation. The figure shows pGADT7-Hpx (HuH-7) translated lysate showing expressed Hpx protein corresponding to expected size of 53 kDa. The relative positions of the protein molecular weight marker and the sizes in kDa are indicated to the left of the panel.
3.1.2.2.1. **Histidine pull-down assay**

To synthesize Hpx protein *in vitro*, the Hpx cDNA subcloned into the pSGI expression vector was transcribed with T7 RNA polymerase and then was translated with a rabbit reticulocyte lysate. A 52 kDa protein of expected size was the most prominent translated product (Fig. 3.1.12A).

![Histidine pull-down assay](image)

**Fig. 3.1.12. Histidine pull-down assay.** A, Hpx protein expression was verified by *in vitro* coupled transcription-translation. The figure shows pSGI-Hpx translated lysate showing expressed Hpx protein corresponding to expected size of 52 kDa. The relative positions of the protein molecular weight marker and the sizes in kDa are indicated to the left of the panel. B, Equal amount of *in vitro* expressed radio labeled $^{35}$S Hpx was added to Ni-NTA beads (lane 1), to beads on which His$_6$-ORF3 protein is immobilized (lane 2) or to control beads (lane 3). Lane 4 is the amount of $^{35}$S Hpx used in each reaction. Samples were resolved by 8% SDS-PAGE and bands were detected by fluorography.

His$_6$-ORF3 protein was expressed in *E. coli* BL21 (DE3) cells and purified on Ni-NTA beads as detailed under “materials and methods”, pg. 61. The ORF3 bound, control and only beads were used directly for histidine pull-down assay. The beads were washed three times in binding buffer. To the washed beads, resuspended in 500 µl binding buffer, 5 µl of lysate containing $^{35}$S labeled *in vitro* translated Hpx protein was added. Beads were incubated for 1 hr at 4°C with constant shaking. The beads were again washed and bound protein complexes were analyzed by adding 20 µl of 2X SDS sample buffer, boiling for 10 min and then analyzing by SDS-PAGE followed by fluorography. The Hpx protein (Fig. 3.1.12B, lane 4) was recovered by the Ni-NTA-His$_6$-ORF3 fusion protein (lane 2). Hpx was not recovered by Ni-NTA alone (lane 1) or by control beads (lane 3), indicating that Hpx interacts specifically.
with the ORF3 portion of the Ni-NTA-His$_6$-ORF3 fusion protein. Although Hpx protein bound to Ni-NTA-His$_6$-ORF3 (lane 2) represented 20% of the total input (lane 4), the binding was reproducible in three independent experiments. This relatively low percentage of binding could be due to sub-optimal binding conditions, an absence of necessary cofactors, or the failure of translated Hpx to achieve an authentic native conformation or modification.

3.1.2.2.2. Interaction between ORF3 and Hpx proteins by in vitro mixing

Similar mixing experiments were performed with COS-1 cell extracts in place of in vitro translated Hpx. Hpx was cloned with a C-terminal EYFP tag in mammalian expression vector pEYFN1. First the expression of EYFP-Hpx was checked by transfecting COS-1 cells with the pEYFPNI-Hpx construct. EYFP-Hpx protein of expected size, ~90 kDa, was specifically immunoprecipitated with anti-GFP antibody (Fig. 3.1.13A, lane 2) and not with preimmune sera (lane 1). EYFP-Hpx expression was also checked by fluorescence microscopy (Fig. 3.1.13B).

![Image](image.png)

**Fig. 3.1.13. Verification of EYFP-Hpx protein expression.** A, COS-1 cells were transfected with EYFPNI-Hpx expression construct and metabolically labeled. Half of the cell lysate was immunoprecipitated with preimmune sera (lane 1) and the other half with anti-GFP antibody (lane 2). Samples were resolved by SDS-PAGE and band detected by fluorography. The relative positions of the protein molecular weight marker and the sizes in kDa are indicated to the left of the panel. B, The image shows expression of EYFP-Hpx in transfected COS-1 cells as visualized under a fluorescence microscope.

In parallel, ORF3 protein was synthesized in vitro, from pSGI-ORF3 expression construct by in vitro coupled transcription-translation (Fig. 3.1.14A, lane 1). The expected 13.5 kDa ORF3 protein and ~28 kDa ORF3 dimer were specifically immunoprecipitated with anti-ORF3 antibody (lane 2) and not with pre-immune sera (lane 3). The EYFP-Hpx construct was then expressed in COS-1 cells and
immunoprecipitated using anti-GFP antibody. After washing three times with immunoprecipitation (IP) buffer, the EYFP-Hpx protein bound to protein A sepharose beads was mixed with $^{35}$S labeled \textit{in vitro} translated ORF3 protein, incubated for 4 hr at 4°C and the beads were washed three times in IP buffer. Any ORF3 protein that associated with Hpx was then determined by fluorography. ORF3 was recovered with EYFP-Hpx (Fig. 3.1.14B, \textit{lane 4}). As a control, pEYFPN1 plasmid (expressing EYFP) was also expressed in COS-1 cells and processed simultaneously as above (\textit{lane 2}) or a mock translated lysate (empty vector pSGI used in \textit{in vitro} coupled transcription-translation) was incubated with EYFP-Hpx protein bound to protein A sepharose beads (\textit{lane 3}). The results indicated that ORF3 interacts specifically with the Hpx part of the protein A sepharose-EYFP-Hpx fusion protein. \textit{Lane 1} shows amount of ORF3 protein used in each reaction mix. Although ORF3 protein bound to protein A sepharose-EYFP-Hpx represented ~50% of the total input, the binding was reproducible in three independent experiments. This could be because of the reasons stated before (section, 3.1.2.2.1).

\textbf{Fig. 3.1.14. Verification of ORF3-Hpx interaction by \textit{in vitro} mixing.} \textit{A}, ORF3 protein expression was verified by \textit{in vitro} coupled transcription-translation. The figure shows pSGI-ORF3 translated lysate (\textit{lane 1}) showing expressed ORF3 protein corresponding to expected size 13.5 kDa (monomer) and the ORF3 dimer. The ORF3 monomer and dimer were specifically immunoprecipitated with anti-ORF3 antibody (\textit{lane 2}) but not with preimmune sera (\textit{lane 3}). The relative positions of the protein molecular weight marker and the sizes in kDa are indicated to the left of the panel. \textit{B}, EYFP-Hpx protein bound to protein A sepharose beads was mixed with \textit{in vitro} expressed $^{35}$S ORF3 protein (\textit{lane 4}) or with a mock translated lysate (\textit{lane 3}). pEYFPN1 plasmid was processed simultaneously and mixed with $^{35}$S ORF3 (\textit{lane 2}). \textit{Lane 1} shows amount of $^{35}$S ORF3 used for each reaction. Samples were resolved by 15% SDS-PAGE and ORF3 protein was detected by fluorography. The figure shows the pull-down of $^{35}$S ORF3 protein by \textit{in vivo} expressed EYFP-Hpx.
3.1.2.2.3. Co-immunoprecipitation of ORF3 and Hpx proteins in COS-1 cells

The ORF3-Hpx interaction was also confirmed in a mammalian cell environment. In mammalian cells, proteins were more likely to be in their native conformations and to have the appropriate post-translational modifications; therefore results were more likely to represent biologically significant interactions. COS-1 cells were co-transfected with EYFPN1-Hpx and pSGI-ORF3 (Fig. 3.1.15, lanes 1 and 6) or pSGI-ORF3 alone (lanes 3 and 4) or EYFPN1-Hpx alone (lanes 2 and 5). Equal amount of protein from each sample was immunoprecipitated using anti-ORF3 (lanes 3, 5 and 6) or anti-GFP antibody (lanes 1, 2 and 4). The samples were split into 2 equal halves, one half was resolved by 8% SDS-PAGE (Fig. 3.1.15, upper panel) and the other half was resolved on 15% SDS-PAGE (lower panel) followed by fluorography to detect EYFP-Hpx and ORF3 expression, respectively.

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**Fig. 3.1.15. EYFP-Hpx protein co-precipitates with ORF3 in mammalian cells.** COS-1 cells were transfected with EYFPN1-Hpx (lanes 2 and 5) or pSGI-ORF3 expression constructs alone (lanes 3 and 4) or co-transfected with both (lanes 1 and 6) and metabolically labeled. The cell lysate was immunoprecipitated with anti-ORF3 antibody (lanes 3, 5 and 6) or with anti-GFP antibody (lanes 1, 2 and 4). Half of the sample was resolved by 8% SDS-PAGE (upper panel) and the other half was resolved by 15% SDS-PAGE (lower panel) followed by fluorography to detect EYFP-Hpx and ORF3 expression, respectively. EYFP-Hpx and ORF3 bands are indicated. EYFP-Hpx and ORF3 co-immunoprecipitate using either anti-GFP or anti-ORF3 antibodies (lanes 1 and 6, respectively).

EYFP-Hpx co-immunoprecipitated with ORF3 using anti-ORF3 antibody (upper panel, lane 6). Reciprocally using anti-GFP antibody, ORF3 co-immunoprecipitated with EYFP-Hpx (lower panel, lane 1). As negative control, EFPN1-Hpx transfected cell lysate was immunoprecipitated with ORF3 antibody (lane 5) and vice versa, pSGI-ORF3 transfected cell lysate was immunoprecipitated
with GFP antibody (lane 4). Lanes 2 and 3 are positive controls for EYFP-Hpx and ORF3 expression, respectively.

3.1.2.3. Co-localization and FRET analysis

To detect ORF3-Hpx interaction in vivo and to complement the results of the in vitro interaction assays, we performed co-localization and FRET analysis. Proteins fused to the cyan (ECFP) and yellow (EYFP) colored variants of the EGFP were used as the donor acceptor FRET pair. The ECFP-ORF3 and EYFPN1-Hpx constructs were co-transfected into COS-1 cells and imaged for ECFP (green pseudo color) and EYFP (red pseudo color), as shown in Fig. 3.1.16, panel I and II, respectively. The distribution of ORF3, as observed earlier (Korkaya et al., 2001; Tyagi et al., 2002), was cytoplasmic and displayed punctate staining. The EYFP-Hpx was also cytoplasmic with some perinuclear localization. The ORF3 protein co-localized with Hpx. Effective co-localization resulted in the production of golden yellow color upon superimposition of panel I over II (panel III).

![Figure 3.1.16. ORF3 co-localizes with Hpx in COS-1 cells.](image)

For FRET analysis, a total of 10 cells were imaged over two separate experiments. Fig. 3.1.17 shows a representative image. ECFP-ORF3 and EYP-Hpx expression can be seen in panels I and II, respectively. ORF3 protein co-localized with Hpx, as observed by presence of yellow colored areas in the superimposed image of panel I over panel II (panel III). To make measurements independent of the expression levels of the two fusion proteins, we followed an acceptor photobleach protocol. The MFI from the donor fluorophore (ECFP-ORF3) before and after photobleaching of the acceptor fluorophore (EYFP-Hpx) was recorded and FRET efficiency was calculated. Panel IV shows ECFP-ORF3 before photobleaching of EYFP-Hpx and panel V shows the same cell after photobleaching. MFI at two areas
within the same cell was recorded, one where the two proteins co-localized (marked A, panel IV and V) and another where no co-localization was observed (C). The MFI obtained in the different areas is graphically shown in Fig. 3.1.17, before photobleaching (BP) and after photobleaching (AP). An average percent FRET efficiency of 14% was found in the region of co-localization as opposed to only 1% in the non-colocalized control area. These results provide strong in vivo support for a physical interaction between the ORF3 and Hpx proteins.

**Fig. 3.1.17. FRET analysis of ORF3-Hpx interaction.** COS-1 cells were co-transfected with ECFP-ORF3 and EYFP-Hpx expression constructs. At 48 hr post-transfection, cells were separately imaged for ECFP (panel I, green pseudo color) and EYFP (panel II, red pseudo color). Areas where the two proteins co-localize result in the production of yellow color upon superimposition of panel I over II (panel III). The ECFP images before photobleach (panel IV) and after EYFP photobleaching (panel V) are shown. Histograms of the mean fluorescence intensity (MFI) of ECFP in the area of co-localization (A) and in the region where the two proteins do not co-localize (C) are shown, either before (BP) or after photobleaching (AP) of EYFP. The numbers on the top right hand side of these panels indicate the MFI value of that area. Representative images are shown from a total of 10 cells imaged over two separate experiments.
3.1.2.4. N-terminal hydrophobic domain II of the ORF3 protein binds to the full-length hemopexin protein

To characterize the domains involved in the ORF3-Hpx interaction, an array of deletion mutations were constructed for both ORF3 and Hpx and were cloned into the yeast two-hybrid AD- and BD- vectors. Pair-wise combinations of full-length fusion constructs and deletion mutants of each fusion construct were tested for protein-protein interaction by yeast two-hybrid assay. The strength of these interactions was investigated by measuring the relative β-galactosidase activity and by the ability to grow in medium lacking histidine and adenine in the presence of 0, 5, 10 and 20 mM 3-AT. The latter results indicated the strength of the protein-protein interaction as a function of histidine prototrophy. The two truncations of the Hpx protein that express each half separately, both showed interaction with full-length ORF3 but with strength 2.6 fold less than that of the full-length protein. None of the other deletion mutants showed interaction with full-length ORF3 when tested in the yeast two-hybrid system (Fig. 3.1.18). On the other hand, ORF3 deletion mutants showed difference in their ability to interact with full-length Hpx in the two-hybrid assay. The ORF3 (1-91) and ORF3 (1-77) truncated proteins gave a positive interaction with full-length Hpx in the yeast two-hybrid assay with strength of interaction comparable to that obtained with full-length ORF3 (1-123). On the other hand, ORF3 (83-123) did not interact with full-length Hpx in the assay. These initial experiments suggested that the region of ORF3 from amino acids 1-77 is involved in the interaction. To further narrow down the interaction domain, ORF3 (1-63) was studied. Although a positive two-hybrid result was obtained, the strength of interaction was about 2 fold less compared to that obtained with full-length ORF3 (1-123), as observed from the liquid β-galactosidase assay and ability to grow only up to 10 mM 3-AT. An analysis of these results suggested that amino acids 63 to 77 may be involved in the interaction, however, presence of this region is not sufficient to give a positive interaction in the two-hybrid assay as is evident by no interaction obtained with the deletion construct ORF3 (63-123). ORF3 (33-123) again gave positive result with strength comparable to full-length ORF3. All of the above results indicate that the N-terminal hydrophobic domain II of the ORF3 protein, spanning amino acids 37 to 62, contains the interaction domain. Growth in presence of 3-AT and relative liquid β-galactosidase units are also shown in the Fig. 3.1.18.
Table 3.18. Deletion mapping of the regions in ORF3 and Hpx responsible for interaction, using the yeast two-hybrid system. The deletion mutants constructed for ORF3 and Hpx were cloned into pAS2 (BD-) or pACT2 (AD-) yeast two-hybrid vectors, respectively and tested for their ability to interact with full-length AD-Hpx or BD-ORF3. The first column gives the overview of the deletion mutants that were assayed. The numbers above the boxes represent the first and the last amino acid of the regions included in the truncated protein. All abbreviations used are as described in Fig. 3.1.9. Interaction data obtained with co-transformants that are able to grow in leu-trp-medium is shown. Interactions were assayed for histidine and adenine prototrophy (ability to grow on leu trp his ade medium) in presence of 0, 5, 10 or 20 mM 3-AT. The horizontal bar graph represents relative \( \beta \)-galactosidase units determined from the liquid \( \beta \)-gal assay.
The yeast two-hybrid results were verified *in vivo* in COS-1 cells. The ORF3 protein and its deletion constructs used for expression in mammalian cells have been described under "materials and methods", pg. 56, Table 2.1, and illustrated below in Fig. 3.1.19.

![Domain Diagram](image)

**Fig. 3.1.19. ORF3 deletion constructs.** The ORF3 protein and its deletion mutants assayed for interaction with EYFP-Hpx in vivo by co-immunoprecipitation analysis are illustrated. The two N-terminal hydrophobic domains, Domain I and II and the two C-terminal proline-rich regions (P1 and P2) are shown. The numbers indicate the amino acids at the boundaries of the various domains, regions and mutants.

An analysis of the 123 amino acid sequence of ORF3 shows two N-terminal hydrophobic domains (Domain I and II) and the two C-terminal proline-rich regions (P1 and P2) (Zafrullah *et al.*, 2000; Korkaya *et al.*, 2001). These deletion mutants were co-transfected into COS-1 cells with EYFPN1-Hpx. EYFP-Hpx expression was checked by visualizing cells under a fluorescence microscope. The cells were metabolically labeled and harvested in IP buffer. One-third of the total cell lysate was resolved by 8% SDS-PAGE and immunoblotted with anti-GFP antibody to detect EYFP-Hpx expression (Fig. 3.1.20, lower panel) and the rest of the sample was immunoprecipitated using either ORF3 antibody [for ORF3 (1-123), ORF3 DI del, ORF3 DII del and ORF3 (33-123)] or anti-his tag antibody [for ORF3 (1-77) and ORF3 (1-91)]. The C-terminal ORF3 deletions (1-77) and (1-91) express truncated proteins with an N-terminal fusion of a hexahistidine tag as well as a phage T7 gene 10 epitope (Zafrullah *et al.*, 1997). This design was used to enable the immunoprecipitation of C-terminally deleted versions of ORF3, since the anti-ORF3 rabbit polyclonal antibody used in the study predominantly recognizes the immunodominant region encompassing residues 90-123 (Zafrullah *et al.*, 1997). One half of the immunoprecipitated sample was resolved by 8% SDS-PAGE and
immunoblotted with anti-GFP antibody to detect co-immunoprecipitated EYFP-Hpx (Fig. 3.1.20, upper panel) and the remaining half was resolved by 15% SDS-PAGE followed by fluorography to detect ORF3 expression (Fig. 3.1.20, middle panel).

Fig. 3.1.20. Co-immunoprecipitation assay for ORF3-Hpx interaction. The full-length ORF3 and its deletions were co-transfected into COS-1 cells with EYFPNI-Hpx. Cells were metabolically labeled and then harvested in IP buffer. One-third of the total cell lysate was resolved by 8% SDS-PAGE and immunoblotted with anti-GFP antibody to detect EYFP-Hpx expression (lower panel). The lower panel shows the Hpx fusion protein expression is equal in all the transfected samples. The rest of the sample was immunoprecipitated using anti-ORF3 antibody (lanes 1, 2, 3 and 4) or anti-his tag antibody (lanes 5 and 6). One half of the immunoprecipitated sample was resolved by 8% SDS-PAGE and immunoblotted with anti-GFP antibody to detect co-immunoprecipitated EYFP-Hpx (upper panel) and the remaining half was resolved by 15% SDS-PAGE followed by fluorography to detect ORF3 expression (middle panel). The ORF3 (1-123) and ORF3 (1-91) bands are indicated with an arrowhead (lane 1 and 6, respectively, middle panel). ORF3 domain I deleted (ORF3 DI del), ORF3 domain II deleted (ORF3 DII del), ORF3 (33-123) and ORF3 (1-77) expressed proteins are clearly seen in lanes 2, 3, 4 and 5, respectively. EYFP-Hpx band is indicated.
As expected, the ORF3 full-length protein was able to associate with and hence pull-down Hpx (upper panel, lane 1). The ORF3 truncated proteins including amino acids 33-123, 1-77 and 1-91 and the Domain I deleted construct could also pull-down Hpx (upper panel, lanes 4, 5, 6 and 2, respectively). These results corroborated with the yeast two-hybrid results. Further the ORF3 mutant, in which the Domain II has been specifically deleted, did not co-immunoprecipitate Hpx (upper panel, lane 3). The middle panel shows the expression of the ORF3 protein and its deletions in corresponding samples. The lower panel shows the Hpx fusion protein expression is equal in all the transfected samples. Together, these experiments confirm that the hydrophobic domain II of the ORF3 protein, spanning amino acids 37 to 62, is responsible for interacting with Hpx. There were quantitative differences between the amount of ORF3 protein and its deletions expressed in cells and hence also in the amount of EYFP-Hpx associated with them. The different expression levels of the ORF3 truncations could also be the result of possibly different specificities of the antibodies used to immunoprecipitate the truncated proteins. Densitometry analysis was thus conducted to quantify the amount of protein that was pulled-down. Densitometry analyses were made using ImageJ software V. 1.36b supported by Wayne Rasband (National Institutes of Health, USA) by integrating intensities of all the pixels in the band excluding the background. To normalize for different expression levels, the data is reported as the ratio of Hpx band intensity to that of ORF3 protein. Despite the quantitative differences between Hpx binding to the full-length and mutant ORF3 proteins, the Domain II deleted mutant reproducibly showed no co-immunoprecipitation of Hpx protein.
3.1.3. Analysis and verification of ORF3-Bβ interaction

3.1.3.1. Yeast two-hybrid analysis

In order to confirm the ORF3-Bβ interaction, the Bβ AD/library plasmid was reintroduced into AH109 along with BD-ORF3 (Fig. 3.1.21).

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**Fig. 3.1.21. Yeast two-hybrid analysis of ORF3-Bβ interaction.** Yeast strain AH109 was singly- or co-transformed with empty BD- and AD- vectors or fused BD- and AD- expression constructs of ORF3 and Bβ. AH109 is the untransformed yeast host strain. AD-ORF3 + BD-ORF3 is the positive control co-transformant used in the study. AD-Bβ + BD- is the negative control indicating reporter gene activity is specifically turned on only in presence of ORF3. Yeast cells were plated on synthetic dropout medium lacking leucine and tryptophan, leu<sub>trp</sub><sup>+</sup>, (to select for AD- and BD- vectors) as co-transformation controls and on medium lacking histidine (his<sup>+</sup>) and adenine (ade<sup>+</sup>) to select for yeast two-hybrid interactions at moderate (leu<sub>trp</sub>his<sup>+</sup>) and high (leu<sub>trp</sub>hisade<sup>+</sup>) stringency. 20 mM 3-AT represents leu<sub>trp</sub>hisade medium to which 20 mM 3-amino-1, 2, 4-triazole has been added. Yeast plates were incubated at 30°C for 4 days. β-gal represents the filter lift β-galactosidase assay. The horizontal bar graph represents relative β-galactosidase units determined from the liquid β-gal assay.

The empty BD- and AD- vectors and bait (BD-ORF3) and prey (AD-Bβ) alone or in combination (BD- + AD- and BD- + AD-Bβ) served as negative controls. ORF3 dimerization was used as the positive control. Our results showed that the histidine, adenine and β-galactosidase reporter activity was specifically turned on only when ORF3 and Bβ were present together inside the yeast host, allowing growth on SD-leu<sub>trp</sub>his<sub>ade</sub> and positive filter β-galactosidase assay. A quantitative estimation of the relative strengths of interaction between ORF3 and Bβ in comparison to
controls as judged by liquid β-galactosidase assay is also shown graphically in Fig. 3.1.21. The liquid β-galactosidase level of ORF3-Bβ co-transformants was almost 16 fold higher compared to the negative controls but about 1.5 fold lesser compared to the positive control. Only the ORF3 and Bβ co-transformants and the positive control were able to grow in the presence of 20 mM 3-AT, clearly displaying specificity and strength of the interaction.

The ORF3-Bβ interaction was also verified using the yeast mating protocol. The AH109 strain (mating type a) was transformed with BD-ORF3 and Y187 (mating type α) with AD-Bβ. The two strains were mated and diploids were analyzed for reporter gene activity. All appropriate positive and negative controls were used as above. The results (Fig. 3.1.22) again showed that diploid cells are able to grow on SD-leu·trp·his·ade· only when they harbor both ORF3 and Bβ constructs. Additionally, when Bβ was switched from AD- to the BD- vector and ORF3 from the BD- to the AD- vector (domain swapping), a positive two-hybrid interaction was obtained, indicating that the ORF3-Bβ interaction is independent of the fused domains. To further validate our findings, Bβ full-length cDNA was obtained from Dr. C. M. Redman and cloned in the AD- vector, pGADT7. Protein expression from the full-length clone, pGADT7-BβFL (AD-BβFL), was verified (pg. 118, Fig. 3.1.27). When this construct was co-transformed with BD-ORF3, a positive yeast two-hybrid interaction was observed (Fig. 3.1.22).

3.1.3.2. In vitro analysis

3.1.3.2.1. Histidine pull-down assay

The yeast two-hybrid data was reconfirmed in vitro by histidine pull-down assay. The full-length fibrinogen Bβ cDNA was cloned in the pSGI expression vector. Expression of Bβ protein was first verified by in vitro coupled transcription-translation (Fig. 3.1.23A. lane 1). The in vitro translated protein thus obtained was immunoprecipitated with anti-fibrinogen antibody (lane 3). As a negative control, only vector translated lysate was processed in parallel (lane 2). The 54 kDa Bβ protein band was specifically pulled-down using anti-fibrinogen antibody. The in vitro translated Bβ protein was employed in the histidine pull-down assay as described on pg. 100.
**Results**

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<th>Two-hybrid transformants</th>
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<th>Leu· Trp·</th>
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**Fig. 3.1.22. Results of yeast two-hybrid retest with Bβ.** AH109a (mating type α) and Y187α (mating type α) are two haploid host cells transformed with either BD- or AD- plasmids or fused BD- and AD- expression constructs of ORF3 and Bβ. Mating the two produce diploid cells (separated by slash, /), the co-transformants containing ORF3 and Bβ together, turn on reporter gene activity. The abbreviations used and negative and positive controls are as described in Fig. 3.1.21. A positive two-hybrid interaction was also obtained when the Bβ protein was expressed as a fusion to the BD- vector and checked for interaction with AD-ORF3. BβFL represents Bβ cDNA obtained from another source. Like the library isolate, this clone also gave a positive two-hybrid interaction with ORF3.

Equal volume of radiolabeled Bβ protein was incubated with Ni-NTA beads alone, beads on which bacterially expressed His6-ORF3 protein was immobilized or control beads pre-incubated with vector transformed bacterial lysate. After binding and washing steps, the Bβ protein retained on the beads was analyzed by SDS-PAGE. The Bβ protein (Fig. 3.1.23B, lane 4) was recovered by the Ni-NTA-His6-ORF3 fusion protein (lane 3). Bβ was not recovered by Ni-NTA alone (lane 1) or by control beads (lane 2), indicating that Bβ interacts specifically with the ORF3 part of the Ni-NTA-His6-ORF3 fusion protein. Although Bβ protein bound to Ni-NTA-His6-ORF3 represented 50% of the total input, the binding was reproducible in three independent experiments. This low percentage of binding could be due to imperfect binding conditions, an absence of necessary cofactors, or the failure of translated Bβ to achieve an authentic native conformation or modification.
Fig. 3.1.23. Histidine pull-down assay. A, Bβ protein expression was verified by in vitro coupled transcription-translation. Lane 1 shows pSGI-Bβ (Bβ) translated lysate showing expressed Bβ protein corresponding to expected size of 54 kDa. pSGI (V, lane 2) and pSGI-Bβ (lane 3) translated lysates were immunoprecipitated with polyclonal anti-fibrinogen antibody (Fbg), resolved by 12% SDS-PAGE and bands were detected by fluorography. B, Histidine pull-down assay for ORF3-Bβ interaction. Ni-NTA beads on which hexahistidine-tagged ORF3 protein, expressed in E. coli BL21(DE3) cells is immobilized (ORF3-beads, lane 3), beads bound to vector transformed bacterial lysate (control-beads, lane 2) and Ni-NTA beads alone (beads, lane 1) were incubated with equal amount of in vitro expressed radiolabeled (35S) Bβ protein. Lane 4 is the amount of 35S Bβ protein used in each reaction. Bβ protein retained on Ni-NTA beads was analyzed by 12% SDS-PAGE followed by fluorography. The relative positions of the protein molecular weight marker are indicated and sizes in kDa of the corresponding bands are given to the left of panels A and B.

3.1.3.2.2. Interaction between ORF3 and Bβ proteins by in vitro mixing

Similar mixing experiments were performed with COS-1 cell extracts in place of in vitro translated Bβ. First, the expression of Bβ in mammalian cells was checked by transfecting COS-1 cells with the empty vector pSGI or the pSGI-Bβ expression construct. Fibrinogen Bβ protein of expected size, ~52 kDa was specifically immunoprecipitated with anti-fibrinogen antibody (Fig. 3.1.24A, lane 2) and not with preimmune sera (lane 3). Lane 1 shows the vector transfected control. In parallel, ORF3 protein was synthesized in vitro, from pSGI-ORF3 expression construct by in vitro coupled transcription-translation (as described on pg. 102). The pSGI-Bβ construct was then expressed in COS-1 cells and immunoprecipitated using anti-fibrinogen antibody. After washing three times with immunoprecipitation (IP) buffer, the Bβ protein bound to protein A sepharose beads was mixed with 35S labeled in vitro translated ORF3 protein, incubated for 4 hr at 4°C and the beads were washed.
three times in IP buffer. Any ORF3 protein that associated with Bβ was then determined by fluorography. ORF3 was recovered with Bβ (Fig. 3.1.24B, upper panel, lane 4).

![Figure 3.1.24](image_url)

**Fig. 3.1.24. Verification of ORF3-Bβ interaction by in vitro mixing.** A, Bβ protein expression. COS-1 cells were transfected with pSGI-Bβ expression construct and metabolically labeled. Cell lysate was immunoprecipitated with anti-fibrinogen antibody (lane 2) or with preimmune sera (lane 3). pSGI vector transfected lysate was also immunoprecipitated with anti-fibrinogen antibody (lane 1). Samples were resolved by SDS-PAGE and band detected by fluorography. The relative positions of the protein molecular weight marker and the sizes in kDa are indicated to the left of the panel. An unknown cellular protein cross-reacting with the anti-fibrinogen antibody is marked with an asterisk. B, Bβ protein bound to protein A sepharose beads was mixed with in vitro expressed [35S]ORF3 protein (lane 4) or with a mock translated lysate (lane 2). pSGI-Bβ transfected lysate immunoprecipitated with preimmune sera was processed simultaneously and mixed with [35S]ORF3 (lane 3). Lane 1 shows amount of [35S]ORF3 used for each reaction. Samples were resolved by SDS-PAGE followed by fluorography to detect ORF3 protein (upper panel) and Bβ (lower panel). The figure shows the pull-down of [35S]ORF3 protein by in vivo expressed Bβ.

As a control, pSGI-Bβ transfected lysate was immunoprecipitated with preimmune sera and processed simultaneously as above (lane 3) or a mock translated lysate (empty vector pSGI used in *in vitro* coupled transcription-translation) was incubated with protein A sepharose beads bound to Bβ protein (lane 2). The results indicated that ORF3 interacts specifically with the Bβ part of the protein A sepharose-Bβ complex. Lane 1 shows amount of ORF3 protein used in each reaction mix. The
Results show the fibrinogen Bβ protein immunoprecipitated from the corresponding samples.

### 3.1.3.2.3. Co-immunoprecipitation of ORF3 and Bβ proteins in COS-1 cells

The ORF3-Bβ interaction was also confirmed by co-immunoprecipitation assay in mammalian cells. COS-1 cells were co-transfected with pSGI-Bβ and pSGI-ORF3 (Fig. 3.1.25, lanes 2 and 5) or singly transfected with pSGI-Bβ (lanes 1 and 3) or pSGI-ORF3 (lanes 4 and 6). After metabolic labeling, the cell lysate was immunoprecipitated using anti-fibrinogen antibody (lanes 1, 5 and 6) or anti-ORF3 antibody (lanes 2, 3 and 4). The immunoprecipitated samples were split into two equal halves, one half was analyzed using 8% SDS-PAGE (Fig. 3.1.25, upper panel) and the other half by 15% SDS-PAGE (lower panel) followed by fluorography to detect fibrinogen Bβ and ORF3 expression, respectively.

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**Fig. 3.1.25.** Bβ co-immunoprecipitates with ORF3 in COS-1 cells. COS-1 cells were transfected with pSGI-Bβ (lanes 1 and 3) or pSGI-ORF3 (lanes 4 and 6) expression constructs alone or co-transfected with both (lanes 2 and 5) and metabolically labeled. Cell lysate was immunoprecipitated with anti-ORF3 antibody (lanes 2, 3 and 4) or anti-fibrinogen antibody (lanes 1, 5 and 6). Half of the sample was resolved by 8% SDS-PAGE (upper panel) and the other half was resolved by 15% SDS-PAGE (lower panel) followed by fluorography to detect Bβ and ORF3 expression, respectively. Bβ and ORF3 bands are indicated. Bβ co-immunoprecipitates with ORF3 using anti-ORF3 antibody (lane 2) and reciprocally ORF3 co-immunoprecipitates with Bβ using anti-fibrinogen antibody (lane 5). An unknown cellular protein cross-reacting with the anti-fibrinogen antibody is marked with an asterisk.

Results of the co-immunoprecipitation study showed that Bβ co-immunoprecipitated with ORF3, using anti-ORF3 antibody (lane 2) and reciprocally, using anti-fibrinogen antibody, ORF3 co-immunoprecipitated with Bβ (lane 5). As a negative control, pSGI-Bβ transfected cell lysate was immunoprecipitated with ORF3.
antibody (lane 3) and vice versa (lane 6), showing no cross-reactivity. Lanes 1 and 4 are positive expression controls for Bβ and ORF3, respectively.

3.1.3.2.4. Co-immunoprecipitation of ORF3 and Bβ proteins in HuH-7 cells

To show the association of ORF3 with endogenous fibrinogen Bβ, co-immunoprecipitation assay was conducted in the human hepatoma cell line, HuH-7. These cells were transfected with empty pSGI vector (Fig. 3.1.26, V, lane 1) or with pSGI-ORF3 expression construct (O3, lanes 2 and 3). At 44 hr post-transfection, cells were metabolically labeled and harvested in immunoprecipitation buffer. Equal amount of protein from each sample was immunoprecipitated using anti-ORF3 antibody. The immunoprecipitated samples were split into two halves, one half was analyzed by 8% SDS-PAGE (upper panel) and immunoblotted with anti-fibrinogen antibody to detect co-immunoprecipitated endogenous Bβ protein and the other half by 15% SDS-PAGE (bottom panel) followed by fluorography to detect ORF3 expression.

![Image of gel electrophoresis](image)

**Fig. 3.1.26. Endogenous fibrinogen Bβ co-immunoprecipitates with ORF3 in HuH-7 cells.** HuH-7 cells were transfected with empty vector (V, lane 1) or pSGI-ORF3 expression construct (O3, lanes 2 and 3) and metabolically labeled. Cell lysate was immunoprecipitated with monoclonal anti-ORF3 antibody (lanes 1 and 2). One set of sample was first immunodepleted with anti-ORF3 antibody and again immunoprecipitated with anti-ORF3 antibody (lane 3). One half of the immunoprecipitated sample was resolved by 8% SDS-PAGE and immunoblotted with anti-fibrinogen antibody to detect co-immunoprecipitated Bβ (upper panel) and the remaining half was resolved by 15% SDS-PAGE followed by fluorography to detect ORF3 expression (lower panel).

The ORF3 protein could co-precipitate endogenous Bβ (upper panel, lane 2). In order to further confirm the specificity of the interaction, one set of sample was
first immunodepleted with anti-ORF3 antibody. This was done to deplete ORF3 protein from the sample but not to completion. The resulting supernatant was again immunoprecipitated using anti-ORF3 antibody and immunoblotted with anti-fibrinogen antibody. As expected, both ORF3 and fibrinogen band intensities were significantly reduced (lane 3). The co-immunoprecipitation studies confirmed that ORF3 associates with fibrinogen Bβ. One interesting observation made from co-immunoprecipitation assay in HuH-7 cells was that ORF3 pulled-down specifically the Bβ chain and not Aα or γ chains of fibrinogen, which, if present, would have been detected by immunoblotting with anti-fibrinogen antibody.

3.1.3.3. ORF3 interacts specifically with fibrinogen Bβ chain

In order to validate the observations that ORF3 interacts only with fibrinogen Bβ and not with its Aα or γ chains, we studied the interaction in yeast and mammalian cells. The Aα, Bβ and γ cDNAs were obtained in the mammalian expression vector pRSV-Neo from Dr. C. M. Redman. The cDNAs were first cloned into yeast two-hybrid vector pGADT7 and gene expression for each construct was confirmed by in vitro coupled transcription-translation. The pGADT7-Aα, pGADT7-Bβ and pGADT7-γ translated lysates were analyzed by SDS-PAGE (Fig. 3.1.27, lanes 1, 2 and 3, respectively). Aα, Bβ and γ chain proteins corresponding to the calculated size of 72, 55 and 49 kDa were obtained, respectively.

![Fig. 3.1.27. In vitro expression of Aα, Bβ and γ proteins. Aα, Bβ and γ protein expression was verified by in vitro coupled transcription-translation. pGADT7-Aα (lane 1), pGADT7-Bβ (lane 2) and pGADT7-γ (lane 3) translated lysates were resolved by 8% SDS-PAGE followed by fluorography. The Aα, Bβ and γ proteins corresponding to expected size of 72, 55 and 49 kDa, respectively are indicated. The relative positions of the protein molecular weight marker are also indicated and sizes in kDa of the corresponding bands are given to the left of the panel.](image-url)
3.1.3.3.1. Yeast two-hybrid analysis

The yeast two-hybrid expression constructs for Aα, Bβ and γ were co-transformed in AH109 with empty BD- vector or the BD-ORF3 (pAS2-ORF3) construct (Fig. 3.1.28). The co-transformants were assayed for two-hybrid interaction and it was found that only ORF3 and Bβ co-transformants grew on SD-leu’trp’his’ade’ plates up to 20 mM 3-AT and showed positive filter β-galactosidase assay.

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<th>Two-hybrid transformants</th>
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<th>Leu’ Trp’ His’</th>
<th>Leu’ Trp’ His’ Ade’</th>
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**Fig. 3.1.28.** ORF3 interacts specifically with Bβ protein and not with Aα and γ in a yeast two-hybrid assay. The abbreviations used are as described for Fig. 3.1.21. Co-transformants with empty BD- vector served as negative control. Co-transformants containing ORF3 and Bβ together, turn on reporter gene activity.

3.1.3.3.2. Co-immunoprecipitation assay in COS-1

The two-hybrid data was verified by co-immunoprecipitation in COS-1 cells. pRSV-Neo-Aα, Bβ and γ expression constructs were transfected in COS-1 cells (Fig. 3.1.29, *lanes* 1, 2 and 3, respectively) or co-transfected with pSGI-ORF3 (Aα and ORF3, *lane* 4; Bβ and ORF3, *lane* 5; γ and ORF3, *lane* 6). Metabolically labeled cell lysate was immunoprecipitated using anti-fibrinogen antibody (*lanes* 1-3) to verify protein expression. For co-immunoprecipitation, anti-ORF3 antibody (*lanes* 4-6) was used. Although, ORF3 expression was comparable in all samples (*bottom panel, lanes* 4-6), only fibrinogen Bβ efficiently co-immunoprecipitates with ORF3 (*lane* 5). A very faint band corresponding to fibrinogen γ chain can be seen in *lane* 6.
ORF3 co-immunoprecipitates specifically the fibrinogen Bβ protein from transfected cells. COS-1 cells were transfected with pRSV-Neo-Aα, pRSV-Neo-Bβ and pRSV-Neo-γ expression constructs singly (lanes 1, 2 and 3, respectively) or co-transfected with pSGI-ORF3 (lanes 4, 5 and 6, respectively) and metabolically labeled. Cell lysates were immunoprecipitated with anti-fibrinogen antibody (upper panel, lanes 1, 2 and 3) to detect Aα, Bβ and γ expression. The co-transfected samples were immunoprecipitated with anti-ORF3 antibody. One half of the immunoprecipitated sample was resolved by 8% SDS-PAGE and the remaining half was resolved by 15% SDS-PAGE followed by fluorography to detect co-immunoprecipitated fibrinogen chains (top panel) and ORF3 expression (bottom panel), respectively. Aα, Bβ, γ and ORF3 protein bands are indicated.

3.1.3.4. Co-localization of ORF3 with endogenous fibrinogen

Having established the ORF3-Bβ interaction, the localization of the two proteins was subsequently observed in HuH-7 cells. HuH-7 cells cultured on cover slips were transfected with pSGI-ORF3 expression construct. At 24 hr post-transfection, cells were fixed and processed for immunofluorescence assay. Cells were first dually labeled with polyclonal anti-fibrinogen (which will label all three chains of fibrinogen) and monoclonal anti-ORF3 antibody. Primary antibody against fibrinogen and ORF3 was detected with anti-rabbit secondary Alexa Fluor 488- (Fig. 3.1.30, panels I and IV) and anti-mouse secondary Alexa Fluor 594-conjugated antibody (panels II and V), respectively. Distribution of ORF3, as observed earlier was cytoplasmic and displayed punctate staining. The endogenous fibrinogen was cytoplasmic in distribution with distinct perinuclear localization, possibly the ER and Golgi complex, characteristic of secretory proteins. In the merged images (panels III and V), distinct golden yellow stained regions were observed indicating co-localization of ORF3 with fibrinogen in these areas.
Fig. 3.1.30. Co-localization of endogenous fibrinogen with ORF3 in liver cells. HuH-7 cells grown on cover slips were transfected with pSGI-ORF3 and at 24 hr post-transfection were doubly labeled with polyclonal anti-fibrinogen and monoclonal anti-ORF3 antibody. Fibrinogen and ORF3 primary antibody were detected with anti-rabbit secondary Alexa Fluor 488- and anti-mouse secondary Alexa Fluor 594-conjugated antibody, respectively. Separate images were acquired showing fibrinogen distribution (panel I and IV) and ORF3 distribution (panel II and V). Regions of co-localizations are seen in yellow in the Merge panels (panels III and VI).

3.1.3.5. FRET measurements of the ORF3-Bβ interaction

To detect protein-protein interactions in vivo and to complement the results of the in vitro interaction assays and yeast two-hybrid results we used FRET. Due to high transfection efficiency and higher protein expression levels in COS-1, this cell line was used to assay ORF3-Bβ interaction by FRET. ORF3 and Bβ proteins fused to the enhanced cyan (ECFP) and enhanced yellow (EYFP), respectively, colored variants of the enhanced green fluorescent protein (EGFP) were used as the donor-acceptor FRET pair. Expression of EYFP-Bβ was first checked by transfecting COS-1 cells with EYPN1-Bβ expression construct. The metabolically labeled cell lysate was immunoprecipitated with preimmune sera (Fig. 3.1.31, lane 1), anti-GFP antibody (lane 2) or anti-fibrinogen antibody (lane3). Bβ protein of expected size, ~90 kDa was specifically immunoprecipitated with anti-GFP and anti-fibrinogen antibody.

COS-1 cells cultured on cover slips were co-transfected with ECFP-ORF3 and EYFP-Bβ expression constructs. At 48 hr post-transfection cells were fixed and imaged for ECFP (green pseudo color) and EYFP (red pseudo color) as shown in Fig. 3.1.32, panels I and II, respectively.
Fig. 3.1.31. EYFP-Bβ protein expression. A, COS-1 cells were transfected with EYFPN1-Bβ expression construct and metabolically labeled. Cell lysate was immunoprecipitated with preimmune sera (lane 1), anti-GFP antibody (lane 2) or with anti-fibrinogen antibody (lane 3). Samples were resolved by SDS-PAGE and band detected by fluorography. The relative positions of the protein molecular weight marker and the sizes in kDa are indicated to the left of the panel.

ORF3 protein co-localized with Bβ, as observed by presence of yellow colored areas in the superimposed image of panel I over panel II (panel III). To make FRET measurements independent of the expression levels of the two fusion proteins, an acceptor photobleach protocol was followed. The MFI from the donor fluorophore (ECFP-ORF3) before and after photobleaching of the acceptor fluorophore (EYFP-Bβ) was recorded and FRET efficiency was calculated. Panel IV shows ECFP-ORF3 before photobleaching of EYFP-Bβ and panel V shows the same cell after photobleaching. MFI at four areas within the same cell was recorded, three areas (marked 1, 2, and 3, panel IV and V) where the two proteins showed co-localization and a control area (C) where no co-localization was observed. The MFI obtained in the different areas is graphically shown in Fig. 3.1.32, before photobleaching (BP) and after photobleaching (AP). An average percent FRET efficiency of 30 ± 12.7 was found in the regions of co-localization as opposed to 5.4 ± 3.38 in control area. The difference in pixel intensity of ECFP, before and after photobleaching of EYFP in the areas of co-localization and the regions where the two proteins do not co-localize when compared for 10 independent observations over two separate experiments, showed high levels of significance with a p value of 1.3 x 10⁻⁶. The presence of FRET indicated an actual protein-protein interaction in vivo.
**Fig. 3.1.32. FRET analysis of ORF3-Bβ interaction.** COS-1 cells were co-transfected with ECFP-ORF3 and EYFP-Bβ expression constructs. At 48 hr post-transfection, cells were separately imaged for ECFP (panel I, green pseudo color) and EYFP (panel II, red pseudo color). Areas where the two proteins co-localize result in the production of yellow color upon superimposition of panel I over II (panel III). The ECFP images before photobleach (panel IV) and after EYFP photobleaching (panel V) are shown. Histograms of the mean fluorescence intensity (MFI) of ECFP in the areas of co-localization (1, 2 and 3) and in the region where the two proteins do not co-localize (C) are shown, either before (BP) or after photobleaching (AP) of EYFP. The numbers on the top right hand side of these panels indicate the MFI value of that area. Representative images are shown from a total of 10 cells imaged over two separate experiments.
3.1.3.6. Mapping the domains involved in ORF3 and fibrinogen Bβ interaction

To characterize the domains involved in the ORF3-Bβ interaction, an array of deletion mutations were constructed for both ORF3 and Bβ and were cloned into the yeast two-hybrid AD- and BD- vectors. Pair-wise combinations of full-length fusion constructs and deletion mutants of each fusion construct were tested for protein-protein interaction by yeast two-hybrid assay. The strength of these interactions was investigated by measuring the relative β-galactosidase activity and by the ability to grow in medium lacking histidine and adenine in the presence of 0, 5, 10 and 15 mM 3-AT. The latter results indicated the strength of the protein-protein interaction as a function of histidine prototrophy.

The two truncations of the Bβ protein that express each half separately, both showed interaction with full-length ORF3. The N-terminal half of Bβ encoding amino acids 1-266 interacted with ORF3 as strongly as did the full-length protein. However, the C-terminal half encoding amino acids 267-466 interacted but with strength 2 fold less than that of the full-length protein. None of the other deletion mutants showed interaction with full-length ORF3 when tested in the yeast two-hybrid system (Fig. 3.1.33). On the other hand, ORF3 deletion mutants showed difference in their ability to interact with full-length Bβ in the two-hybrid assay. The ORF3 (1-91) and ORF3 (1-77) C-terminal truncated proteins interacted with full-length Bβ in the yeast two-hybrid assay, but with strength 1.7 fold to that obtained with full-length ORF3 (1-123). C-terminal truncations beyond amino acid 77 [ORF3 (1-63)] however, completely abolished the interaction. Additionally, ORF3 (83-123) also did not interact in the two-hybrid assay. These initial experiments suggested that the region of ORF3 from amino acids 63-77 is critical for the interaction. The C-terminal amino acids beyond amino acid 77 possibly contribute to the strength of the interaction, however, presence of this region is not sufficient to give a positive two-hybrid result as evident by no interaction obtained with the deletion construct ORF3 (83-123). This observation was further substantiated by the fact that ORF3 (63-123) interacted with Bβ with strength comparable to full-length ORF3-Bβ interaction. ORF3 (33-123) also interacted with strength comparable to full-length ORF3. All of the above results indicate that the C-terminal half of ORF3 from amino acids 63-123 contains the interaction domain, and possibly the presence of amino acids 63-77 of ORF3 is critical for the association.
Fig. 3.1.33. Deletion mapping of the regions in ORF3 and Bβ responsible for interaction, using the yeast two-hybrid system. The deletion mutants constructed for ORF3 and Bβ were cloned into pAS2 (BD-) or pACT2 (AD-) yeast two-hybrid vectors, respectively; and tested for their ability to interact with full-length AD-Bβ or BD-ORF3. The first column gives the overview of the deletion mutants that were assayed. The numbers above the boxes represent the first and the last amino acid of the regions included in the truncated protein. All abbreviations used are as described in Fig. 3.1.21. Interaction data obtained with co-transformants that are able to grow in leu-trp-his-ade- medium is shown. Interactions were assayed for histidine and adenine prototrophy (ability to grow in leu-trp-his-ade- medium) in presence of 0, 5, 10 or 15 mM 3-AT. The horizontal bar graph represents relative β-galactosidase units determined by the liquid β-gal assay.
The yeast two-hybrid results were verified \textit{in vivo} in COS-1 cells. The ORF3 protein and its deletion constructs used for expression in mammalian cells have been described before, pgs. 56 and 108. These deletion mutants were co-transfected into COS-1 cells with pSGI-Bβ. The cells were metabolically labeled and harvested in IP buffer. Equal protein from each sample was immunoprecipitated using anti-ORF3 antibody [for ORF3 (1-123) and ORF3 (33-123)] or anti-his tag antibody [for ORF3 (1-77) and ORF3 (1-91)]. One half of the immunoprecipitated sample was resolved by 8% SDS-PAGE and immunoblotted with anti-fibrinogen antibody to detect co-immunoprecipitated Bβ (Fig. 3.1.34, upper panel) and the remaining half was resolved by 15% SDS-PAGE followed by fluorography to detect ORF3 expression (Fig. 3.1.34, lower panel).

\textbf{Fig. 3.1.34. Co-immunoprecipitation assay to map ORF3 interaction domain.} The full-length ORF3 and its deletion mutants were co-transfected into COS-1 cells with pSGI-Bβ. Cells were metabolically labeled and then harvested in IP buffer. Equal protein from each sample was immunoprecipitated using anti-ORF3 antibody (lanes 1 and 2) or anti-His tag antibody (lanes 3 and 4). One half of the immunoprecipitated sample was resolved by 8% SDS-PAGE and immunoblotted with anti-fibrinogen antibody to detect Bβ (upper panel), the remaining half was resolved by 15% SDS-PAGE followed by fluorography to detect ORF3 expression (lower panel). The ORF3 (1-77) and ORF3 (1-91) bands are indicated with an arrowhead (lane 3 and 4, respectively, lower panel). ORF3 (1-123) and ORF3 (33-123) expressed proteins are clearly seen in lanes 1 and 2, respectively. Bβ band is indicated.
As expected, the ORF3 full-length protein was able to associate with and hence pull-down Bβ (*upper panel, lane 1*). The ORF3 truncated proteins including amino acids 33-123, 1-77 and 1-91 could also pull-down Bβ (*upper panel, lanes 2, 3 and 4, respectively*), but to different extents. These results corroborated with the yeast two-hybrid results. The *lower panel* shows the expression of the full-length ORF3 protein and its deletions in corresponding samples. There were quantitative differences between the amount of ORF3 protein and the different deletions expressed in cells and hence also in the amount of Bβ associated with them. Densitometry analysis was done to quantify the amount of protein that was pulled-down. To normalize for different expression levels, the data is reported as the ratio of Bβ band intensity to that of ORF3 protein.
Aim: To establish the physiological relevance of the ORF3-Bβ interaction

Elucidate the mechanism of ORF3 dependent decrease in fibrinogen secretion from human hepatoma cells

In the preceding section, ORF3 interaction with fibrinogen Bβ protein was established. Previous studies in the lab have shown that ORF3 protein promotes secretion of α1 microglobulin, whose inhibitory effects on the immune system are well documented, supporting the hypothesis that it creates an immunosuppressed environment around the infected hepatocytes (Tyagi et al., 2004). Fibrinogen is the key player in maintaining hemostasis and has been proposed to be one of the key regulators of the inflammatory response (Flick et al., 2004a, 2004b). ORF3 interaction with fibrinogen Bβ was thus of considerable interest to us. We wanted to establish the physiological relevance of this interaction in tissue culture. Initial studies with immunofluorescence assay of ORF3 transfected HuH-7 cells showed a notable phenomenon. In ORF3 expressing liver cells, 48 hr post-transfection, it was observed that either endogenous fibrinogen could not be detected at all or in few cells reduced levels were detected. Furthermore, metabolic labeling of ORF3 transfected HuH-7 cells showed drastic reduction in the secreted fibrinogen level when compared with vector transfected control. Experiments were thus conducted to elucidate the mechanism of ORF3 mediated decrease in fibrinogen secretion from human hepatoma cells and to establish its physiological relevance. This section includes the studies conducted to explore the fate of fibrinogen in ORF3 transfected cells.

3.2.1. Hepatocytes expressing ORF3 protein show a shift in distribution of fibrinogen

HuH-7 cells transfected with the pSGI-ORF3 expression construct were processed for immunofluorescence. At 48 hr post-transfection, cells dually labeled for ORF3 and fibrinogen, showed a notable phenomenon. In ORF3 expressing liver cells, either endogenous fibrinogen could not be detected at all or in few cells reduced levels were detected (characterized as cells with less intense staining). ORF3 non-expressing cells, on the other hand, stained well for fibrinogen showing a
characteristic perinuclear staining. Fig. 3.2.1 shows a representative field at the 48 hr time-point. Panel a shows fibrinogen distribution in HuH-7 cells and panel b shows ORF3 expressing cells in the same field. Panel c is the merged image of a over b. To ensure that this observed phenomenon was specifically due to ORF3 expression, HuH-7 cells were transfected with pSGI-ORF2 construct. Panel e shows a single cell expressing ORF2. ORF2 distribution was cytoplasmic as reported earlier (Zafrullah et al., 1999). The same cell when imaged for fibrinogen (panel d) showed the characteristic perinuclear staining. Panel f is the merged image, showing no co-localization of ORF2 and fibrinogen. Since fibrinogen is a secretory protein, we studied its co-localization with ER and Golgi markers in presence and absence of ORF3. Cells were transfected with DsRed-ER construct without ORF3 (panel h) and alternatively with the CFP-ORF3 expression construct (panel k). Panel l shows CFP-ORF3 expression in the same cell shown in panel k. Fibrinogen distribution was imaged in the cells (panel g) in the same field as panel h and the merged image (panel i) of g over h showed co-localization of fibrinogen with ER marker. However, in cells expressing ORF3, fibrinogen was no longer detectable in the ER (panel j). Similarly, HuH-7 cells were transfected with YFP-Golgi without ORF3 (panel n) or with the CFP-ORF3 expression construct (panel q). Panel r shows CFP-ORF3 expression in the same cell shown in panel q. As observed with ER marker, fibrinogen co-localized with Golgi marker (panel o), but in cell expressing ORF3, no endogenous fibrinogen was detected (panel p). It was clear from these experiments that endogenous fibrinogen distribution radically changes in the presence of ORF3. To further understand the phenomenon, HuH-7 cells were transfected with pSGI-ORF3 and cells were imaged for fibrinogen and ORF3 expression at 12, 24, 36 and 48 hr post-transfection. Data obtained is given in Table 3.2.1. 200 cells were imaged at each time-point and the numbers of cells expressing ORF3 were counted. With increasing time, the number of cells expressing ORF3 gradually increased. At 48 hr post-transfection, 110 cells out of 200 HuH-7 cells counted showed ORF3 expression, indicating transfection efficiency of close to 60%. The ORF3 expressing cells were then imaged for fibrinogen staining. Some ORF3 expressing cells showed presence of endogenous fibrinogen but the staining was less intense compared to surrounding ORF3 non-expressing cells. A representative cell is shown in Fig. 3.2.2.
Fig. 3.2.1.

(Figure legend on pg. 131)
Fig. 3.2.2. Endogenous fibrinogen and ORF3 staining in HuH-7. HuH-7 cells grown on cover slips were transfected with pSGI-ORF3 and at 48 hr post-transfection, were doubly labeled with polyclonal anti-fibrinogen and monoclonal anti-ORF3 antibody. Fibrinogen and ORF3 primary antibody were detected with anti-rabbit secondary Alexa Fluor 488- and anti-mouse secondary Alexa Fluor 594-conjugated antibody, respectively. Separate images were acquired showing fibrinogen distribution (panel I) and ORF3 distribution (panel II). Regions of co-localizations are seen in yellow in the Merge panel (panel III). Reduced level of fibrinogen staining in ORF3 expressing cell can clearly be seen.

Since this could be a staining artifact, only cells visually showing complete clearing of endogenous fibrinogen were counted. The results indicated that even at 12 and 24 hr post-transfection, ~75% ORF3 transfected cells showed no endogenous fibrinogen staining, which increased to 90% at 48 hr. Since a significant population of ORF3 expressing cells showed this phenomenon, we ruled out it to be an experimental artifact. It was further established that the fibrinogen clearing from the cells was a phenomenon specific to ORF3. All pSGI-ORF2 transfected cells imaged for fibrinogen staining 48 hr post-transfection showed distinct perinuclear fibrinogen staining.

These observations gave preliminary indication that the intracellular processing and/or secretion of fibrinogen was altered in the presence of the ORF3.
protein within the hepatocytes.

**Table 3.2.1. Statistical analysis of ORF3-mediated disappearance of fibrinogen staining**

<table>
<thead>
<tr>
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<th>Vector</th>
<th>ORF3 12 hr</th>
<th>ORF3 24 hr</th>
<th>ORF3 36 hr</th>
<th>ORF3 48 hr</th>
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<tr>
<td>Number of cells expressing ORF3</td>
<td>44 ± 10.1</td>
<td>62 ± 7.8</td>
<td>96 ± 5.2</td>
<td>110 ± 8.6</td>
<td></td>
</tr>
<tr>
<td>Number of cells not stained for fibrinogen (Percentage)</td>
<td>33 ± 11 (75)</td>
<td>46 ± 5.8 (74)</td>
<td>86 ± 6.6 (89)</td>
<td>102 ± 8.1 (92)</td>
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</table>

HuH-7 cells were transfected with pSGI-ORF3 and visualized by fluorescence microscopy. Anti-mouse Alexa 594 was used to detect primary antibody against ORF3. Fibrinogen distribution in the cells was visualized with anti-rabbit Alexa 488. A total of 200 ORF3-transfected cells were counted at respective time points and first scored for ORF3 expression and then the same cells were imaged for presence or absence of fibrinogen staining. Only cells showing complete disappearance of detectable fibrinogen staining were counted as positives. Vector transfected cells, were taken as control. Data shown are ± S.E. from three independent sets of experiments.

3.2.2. ORF3 expressing hepatocytes secrete less fibrinogen

Since fibrinogen could not be detected in ORF3 expressing HuH-7 cells, one possibility for this could be that fibrinogen secretion was being expedited by ORF3, a phenomenon observed earlier for α₁ microglobulin (Tyagi *et al.*, 2004). Thus the level of secreted fibrinogen was studied from cells expressing ORF3. HuH-7 cells were transfected with empty vector (Fig. 3.2.3A, lane 1) or pSGI-ORF3 expression construct (lane 2). After metabolic labeling, secreted fibrinogen was immunoprecipitated from the medium with anti-fibrinogen antibody. The immunoprecipitated sample was aliquot into two halves. One half was analyzed by SDS-PAGE under reducing conditions (Fig. 3.2.3A, top panel) to detect the three component chains of fibrinogen, Aα, Bβ and γ and the other half was analyzed by non-reducing SDS-PAGE to detect the fibrinogen complex (bottom panel). The cell lysate was immunoprecipitated with anti-ORF3 antibody to detect ORF3 expression (middle panel). A densitometry analysis of the immunoprecipitated bands was done and relative densitometry units were calculated. The relative densitometry units of the three fibrinogen chains in vector and ORF3 transfected cells (top panel) are represented by a bar graph. As is clearly evident, ORF3 transfected cells secreted less fibrinogen compared to vector transfected cells seen by decreased level of all the three
fibrinogen chains (compare lane 1 and 2, top panel). Similarly the level of the fibrinogen complex from ORF3 transfected cells was 2 fold less compared to vector transfected cells (compare lanes 2 and 1, bottom panel).

Fig. 3.2.3. Decreased secretion of fibrinogen from HuH-7 cells expressing ORF3 protein. A, HuH-7 cells were transfected with empty vector (lane 1) or pSGI-ORF3 expression construct (lane 2) and metabolically labeled with 100 μCi 35S cys/m for 3 hr. At 48 hr post-transfection secreted fibrinogen was immunoprecipitated with anti-fibrinogen antibody from the labeling medium. The immunoprecipitated protein was analyzed by SDS-PAGE under reducing conditions (top panel) and non-reducing conditions (bottom panel) followed by fluorography. The cell lysate was immunoprecipitated with anti-ORF3 antibody to see ORF3 expression (middle panel). Relative densitometry units of individual fibrinogen chains Aα, Bβ and γ in vector (V) and ORF3 transfected cells is represented graphically. The graph shows the ± S.E. of relative densitometry units for protein levels of individual fibrinogen chains as examined in three independent sets of experiments. Relative densitometry units of non-reduced fibrinogen complex are given below the corresponding lanes. B, Fibrinogen secretion decreases specifically from ORF3 transfected cells. HuH-7 cells were transfected with pSGI-ORF2 (lane 1), empty vector (lane 2) or pSGI-ORF3 (lane 3). Secreted fibrinogen was immunoprecipitated as described above followed by SDS-PAGE under reducing (top panel) and non-reducing conditions (bottom panel). The cell lysate was immunoprecipitated with anti-ORF2 antibody (lane 1) or anti-ORF3 antibody (lanes 2 and 3) to detect ORF2 and ORF3 expression (middle panel). Relative densitometry units of individual fibrinogen chains are represented graphically as ± S.E. of units as examined in three independent sets of experiments.
To check whether this effect is specific to ORF3, the same set of experiment was carried out in the presence of ORF2. Fig. 3.2.3B, *top panel* shows the autoradiogram of the reduced gel showing Aα, Bβ and γ chains, immunoprecipitated from medium of pSGI-ORF2 (*lane 1*), vector (*lane 2*) and ORF3 (*lane 3*) transfected cells. The relative densitometry units of each band are plotted in a bar graph. The levels of Aα, Bβ and γ proteins were found to be unchanged in ORF2 and vector transfected cells but much less in ORF3 transfected cells. The middle panel shows corresponding cell lysates immunoprecipitated with anti-ORF3 (*lanes 2 and 3*) or anti-ORF2 (*lane 1*) antibody.

### 3.2.3. ORF3 does not alter glycosylation state or the secretory pathway of fibrinogen

Since significant reduction in the levels of secreted fibrinogen in ORF3 transfected cells was observed, we attempted to follow the transit of fibrinogen molecules along the secretory pathway using Endo H treatment. Endo H cleaves the high mannose form of N-linked fibrinogen Bβ and γ glycoproteins, found in the ER and the cis-Golgi (Kornfeld and Kornfeld, 1985; Maley et al., 1989). In the medial-Golgi, these proteins acquire a complex oligosaccharide form, rendering them Endo H resistant (Townsend et al., 1982). HuH-7 cells were transfected with the pSGI-ORF3 expression construct (Fig. 3.2.4, *lanes 1 and 2*) or with vector (*lanes 3 and 4*). After metabolic labeling with 100 μci 35S cys/met for 2 hr, the intracellular and secreted fibrinogen molecules were treated with Endo H as described under "materials and methods". As seen in Fig. 3.2.4, fibrinogen chains present within ORF3 transfected cells (*top panel, lane 2*) have the same sensitivity to Endo H as that of the chains present in vector transfected cells (*lane 4*). The migrational shifts of Bβ and γ chains in *lanes 2 and 4*, indicates that these chains are present predominantly as the high mannose form. The secreted Bβ and γ chains from ORF3 and vector transfected cells (*bottom panel*) are Endo H resistant indicating conversion to a complex oligosaccharide form. This experiment clearly demonstrated that ORF3 is not interfering with fibrinogen Bβ and γ chain glycosylation. Further, it shows that fibrinogen is secreted via the same pathway in both ORF3 and vector transfected cells.
Fig. 3.2.4. Endoglycosidase H treatment of fibrinogen. HuH-7 cells were transfected with empty vector, V (lanes 3 and 4) or pSGI-ORF3 expression construct (lanes 1 and 2) and metabolically labeled with 100 μci 35S cys/met for 3 hr. At 48 hr post-transfection, secreted (bottom panel) and intracellular (top panel) fibrinogen was immunoprecipitated with anti-fibrinogen antibody and the immunoprecipitate was incubated with (lanes 2 and 4) or without (lanes 1 and 3) Endo H as described under "materials and methods". The proteins were then analyzed by 8% SDS-PAGE under reducing conditions followed by fluorography. The positions of Aα, Bβ, and γ protein bands are indicated. The faster mobility of Bβ and γ chains in lanes 2 and 4 in the intracellular fraction (top panel) is due to removal of high mannose oligosaccharides from these N-linked glycoproteins by Endo H.

In order to further confirm that the effect of ORF3 on fibrinogen secretion is not due to interference with the secretory pathway, a pulse-chase assay was conducted to study the kinetics of fibrinogen secretion in the presence and absence of ORF3. Vector and ORF3 transfected HuH-7 cells were pulse-labeled for 10 minutes with 500 μci 35S cys/met and chased in complete medium for 130 min. At the indicated chase periods (20, 40, 70 and 100 minutes), chase media was collected and replaced with fresh medium. Secreted fibrinogen was immunoprecipitated. Fig. 3.2.5 shows the immunoprecipitated protein bands analyzed by reducing SDS-PAGE from vector (top panel) and ORF3 (bottom panel) transfected cells. Fibrinogen assembly commences by incorporation of nascent Bβ chains with pre-existing intracellular pools of Aα and γ chains (Yu et al., 1983, 1984). Thus after metabolically labeling and analysis under reducing conditions, radiolabeled Bβ chain can be detected in the medium first, followed by Aα and γ, which are subsequently used for assembly. Data from pulse-chase experiments showed that radiolabeled Bβ chain from both vector and ORF3 transfected cells reached detectable levels in the medium at 20-40 min of secretion (top and bottom panels, lane 2) followed by appearance of very faint bands of Aα and γ chains in the next 30 min (top and bottom panels, lane 3). This indicated that the
time required for chain assembly and intracellular transport of fibrinogen was not altered by ORF3.

![Chase times](image)

**Fig. 3.2.5. Rate of secretion of fibrinogen.** Rate of secretion of fibrinogen from ORF3 and vector transfected cells is shown. HuH-7 cells were transfected with empty vector (V) or pSGI-ORF3 and pulse labeled with 500 µci [35S]cys/met for 10 min and chase incubated for 130 min. At the indicated chase periods, the incubation medium was collected and replaced with fresh medium. Pulse labeled secreted fibrinogen was immunoprecipitated and analyzed by SDS-PAGE followed by fluorography. Lane 1 shows the radioactive composition of fibrinogen secreted between 0-20 min of the chase period, lane 2 that secreted between 20-40 min, lane 3 that between 40-70 min, lane 4 that between 70-100 min and lane 5 that between 100-130 min.

### 3.2.4. Reduction in intracellular fibrinogen levels in ORF3 expressing hepatocytes

The intracellular levels of fibrinogen synthesized in HuH-7 cells expressing ORF3 was compared with that of vector transfected cells. First, the initial rate of synthesis of the Aα, Bβ and γ chains in vector and ORF3 transfected cells were compared by pulse-labeling vector (Fig. 3.2.6, lanes 1-3) and ORF3 (lanes 4-6) transfected cells for 10, 20 or 30 min and immunoprecipitating the intracellular fibrinogen chains synthesized at these time points. The bands were analyzed by reducing SDS-PAGE. The relative densitometry units of Aα, Bβ and γ chains are represented by line graphs. Bands with lower molecular weights than the γ chains, which may represent incomplete fibrinogen chains, were also detected. In ORF3 transfected cells, after labeling for 10 min, it was found that more Aα chain is synthesized as compared to the vector transfected cells. However, its rate of synthesis was comparable in both when observed over 20 and 30 min. The rate of synthesis of
Bβ chain was also comparable in the ORF3 and vector transfected cells at each time point studied. However, the amount of γ chain synthesized in ORF3 transfected cells over a 30 minute pulse period was lower than the amount synthesized in vector transfected cells at the same time point, although, rate of synthesis of the γ chain, when compared over 10 and 20 min of pulse periods, remained unchanged. The data suggested that the initial rates of synthesis of the fibrinogen chains were not severely affected and were comparable in the ORF3 and vector transfected cells.

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<tr>
<th>Plasmid</th>
<th>V</th>
<th>ORF3</th>
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<td>Pulse</td>
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<td>minutes</td>
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![Graph showing the synthesis rates of fibrinogen chains](image)

**Fig. 3.2.6. Initial rates of synthesis of individual fibrinogen chains.** Initial rates of synthesis of the individual fibrinogen chains in ORF3 and vector transfected cells were studied. HuH-7 cells were transfected with empty vector, V (lanes 1-3) or pSGI-ORF3, ORF3 (lanes 4-6) and pulse labeled with 500 μCi 35S cysteine for 10, 20 or 30 min. Synthesized radioactive fibrinogen chains were immunoprecipitated from the cell lysates and analyzed by SDS-PAGE followed by fluorography. The relative densitometry units of Aα, Bβ and γ chains at each time point are represented by line graphs, where ■ and ▲ symbols represent, vector and ORF3 transfected cells, respectively.
The difference in intracellular level of the individual fibrinogen chains was more pronounced when the levels were compared after labeling for 3 hr, to achieve steady state levels of protein radioactivity (Fig. 3.2.7). To make measurements independent of any difference in secretion processes in the ORF3 and vector transfected cells, the secretory pathway was blocked with monensin. Monensin blocks protein transport beyond the trans-Golgi vesicle resulting in intracellular accumulation of protein (Mollenhauer et al., 1990). HuH-7 cells were transfected with empty vector (V) or pSGI-ORF3 (O3). After metabolic labeling in the presence or absence of monensin, secreted and intracellular fibrinogen was analyzed by reducing SDS-PAGE. The relative densitometry unit of each fibrinogen chain is represented by a bar graph (Fig. 3.2.7).

<table>
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<th>Plasmid</th>
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<th>Secreted</th>
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<td>Monensin</td>
<td>V</td>
<td>V</td>
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![Image](image_url)

**Fig. 3.2.7.** Intracellular fibrinogen level is lower in HuH-7 cells expressing ORF3. HuH-7 cells were transfected with empty vector (V) or pSGI-ORF3 expression construct (O3). Metabolically labeled intracellular and secreted fibrinogen was immunoprecipitated from cell lysates (lanes 1-3) and medium (lanes 4-6) in the presence (+) or absence (-) of monensin. The immunoprecipitated samples were analyzed by SDS-PAGE followed by fluorography. The image is a representative of three independent sets of experiments. The mean densitometry units of individual fibrinogen chains in each lane are represented graphically.
Results thus obtained clearly showed that vector transfected cells secrete fibrinogen, which is effectively blocked by monensin treatment and hence very minute amounts of it were detected in monensin treated cells (compare lanes 5 and 6). Correspondingly, the intracellular levels of the component fibrinogen chains in monensin treated vector transfected cells (lane 2) were more than non-treated cells (lane 1). Under the same conditions, when intracellular levels of fibrinogen chains were compared in monensin treated vector (lane 2) and ORF3 (lane 3) transfected cells, the latter clearly showed lower levels of each one of the fibrinogen chains. These results indicated that either ORF3 downregulates endogenous fibrinogen expression or the stability of fibrinogen is compromised in presence of ORF3.

These results were further substantiated by immunofluorescence assay. Fig. 3.2.8 shows the effect of brefeldin A and monensin on endogenous fibrinogen in the presence of ORF3. Brefeldin A specifically inhibits ER to Golgi transport (Misumi et al., 1986). ORF3 transfected HuH-7 cells were treated with either of the two drugs for 1 hr, to block cellular secretory pathway, before fixing the cells and processing for immunofluorescence assay. ORF3 mediated disappearance of fibrinogen staining from cells was unaffected by brefeldin A (panels I-III) or monensin (panels IV-VI), again suggesting that the ORF3 mediated decrease in fibrinogen levels from cells was not due to any differences in the fibrinogen secretory pathway.

**Fig. 3.2.8.** HuH-7 cells were transfected with pSGI-ORF3 and treated with brefeldin A or monensin, 1 hr before fixing the cells and processing for immunofluorescence assay. Panel I shows fibrinogen distribution in brefeldin A treated cells and panel II shows ORF3 expressing cells in the same field. Panel IV shows fibrinogen distribution in monensin treated cells and panel V shows ORF3 expressing cells in the same field. Panels III and VI are the respective merged images.
3.2.5. Degradation of fibrinogen chains in ORF3 expressing hepatocytes

Since intracellular fibrinogen level is reduced in ORF3 transfected cells, we sought to determine whether ORF3 played a role in the degradation of fibrinogen chains. We thus examined the effect of ORF3 on the rate of degradation of newly synthesized Aα, Bβ and γ chains in ORF3 transfected HuH-7 cells. The vector or ORF3 transfected cells were pulse-labeled with 200 μci 35S cys/met to achieve steady state levels of the radiolabeled fibrinogen chains and then chase incubated for 1, 2, 3, 4, 5 or 6 hr. The amount of intracellular fibrinogen chains at the end of the pulse incubation (indicated by 0 hour, Fig. 3.2.9) and at the indicated chase time points was determined by immunoprecipitation of the cell lysate with anti-fibrinogen antibody followed by SDS-PAGE under reducing conditions and fluorography. The relative densitometry unit of each band was determined and the data was plotted as percentage stability, assuming relative densitometry unit at 0 hour to be 100%. Fig. 3.2.9A and 3.2.9B show the rate of degradation of radiolabeled Aα, Bβ and γ chains in vector and ORF3 transfected cells, respectively. About 50% of Aα chain was degraded in 1 hr, Bβ in 110 min and γ chain in 3 hr. These times were comparable to what have been reported in COS cells, 1 hr, 80 min and 4 hr for Aα, Bβ and γ, respectively (Roy et al., 1992; Xia and Redman, 1999). In HepG2 cells too, γ chain had a half-life of 4 hr (Roy et al., 1992; Xia and Redman, 1999). The rate of degradation of fibrinogen chains was also studied in the presence of monensin to block the secretory pathway. In both vector and ORF3 transfected cells (Fig. 3.2.9C and 3.2.9D, respectively) the estimated half-life of Aα, Bβ and γ chains was over 4 hr for each chain. Danishefsky et al. (1990) reported non-secreted Bβ chain was degraded intracellularly with a half-life of 5 hr in COS-1 cells. Lane 8 in Fig. 3.2.9C and 3.2.9D shows fibrinogen immunoprecipitated from the medium at the end of pulse incubation, showing that monensin effectively blocks fibrinogen secretion. The observed increase in stability of the fibrinogen chains in both vector and ORF3 transfected cells is most likely due to the presence of monensin, the mechanism of which is still unclear.
Fig. 3.2.9. Fibrinogen degradation in presence of ORF3. HuH-7 cells were transfected with empty vector, V (panels A and C) or pSGI-ORF3 (panels B and D). The transfected cells were metabolically labeled with 200 μCi 35S cys/met for 2 hr and then chase incubated for indicated time periods. At the indicated chase periods (0, 1, 2, 3, 4, 5, 6 hr, corresponding to lanes 1, 2, 3, 4, 5, 6, 7, respectively), radioactive fibrinogen was immunoprecipitated from cell lysates and analyzed by reducing SDS-PAGE followed by fluorography. 0 hr represents intracellular fibrinogen at the end of the pulse period. In panels C and D, metabolic labeling and chase incubations were carried out in presence of monensin. Lane 8 in panels C and D is fibrinogen immunoprecipitated from medium at the end of pulse incubation. Aa, Bβ and γ chain protein bands are indicated in all panels. Intensity of individual bands was quantitated by densitometry. Data is represented by a line graph below each panel, where ■, ● and ▲ symbols represent Aa, Bβ and γ chains, respectively. Results are representative of two independent experiments.
3.2.6. Fibrinogen RNA levels are transcriptionally downregulated in ORF3 expressing hepatocytes

Since the degradation of fibrinogen was observed to be unaltered in presence of ORF3, the observed decrease in intracellular and secreted fibrinogen levels were presumed to be due to downregulation of fibrinogen mRNAs in ORF3 expressing hepatocytes. To test this, total RNA was isolated from vector or ORF3 transfected HuH-7 cells and the mRNA levels of the fibrinogen chains were compared by northern hybridization. The blots were hybridized separately with \((\alpha^{32}P)dCTP\) labeled Aα, Bβ and γ cDNA. Results are shown in Fig. 3.2.10. *Lane 1 contains RNA

![Image](image_url)

*Fig. 3.2.10. Transcriptional downregulation of fibrinogen by ORF3.* Northern hybridization was done to compare fibrinogen mRNA levels. HuH-7 cells were transfected with empty vector or pSGI-ORF3. In lane 1, total RNA from vector transfected cells and in lane 2, total RNA from ORF3 transfected cells was run. RNA was transferred to nylon membrane and probed separately with \((\alpha^{32}P)dCTP\) labeled Aα, Bβ and γ cDNA. Methylene blue stained 28S and 18S rRNA is shown as loading control. The relative intensities of the specific bands are graphically represented for each chain in vector (V) and ORF3 transfected cells. The approximate sizes of hybridized mRNA is shown determined by the relative position of the 28S (5.02 kb) and 18S (1.86 kb) rRNA in the methylene blue stained blot.
from vector transfected cells and lane 2 from ORF3 transfected cells, in each panel. A methylene blue stained blot shows integrity and equal loading of RNA in the two lanes. The Aα, Bβ and γ specific mRNAs are indicated in their respective blots and sizes of the mRNA in kb are shown. The relative densitometry units of the bands obtained are compared in the bar graph. As expected, mRNA levels of the three chains were found to be downregulated in ORF3 transfected cells as compared to mock. About 2.5 fold decrease in mRNA levels was observed for Aα and Bβ chains and about 3 fold for γ chain.

The decreases in the mRNA levels of the fibrinogen genes were verified independently by RT-PCR analysis. Fig. 3.2.11 shows the RT-PCR results showing lower mRNA levels of fibrinogen chains Aα, Bβ and γ in ORF3 transfected HuH-7 cells (O3, lanes 1, 3 and 5) compared to vector transfected (V, lanes 2, 4 and 6). The densitometry units of each band in the top panel are indicated. The levels of β-actin mRNA were checked to verify that equal amounts of RNA were used in each reaction. The bottom panel shows amplified β-actin cDNA from the corresponding samples. These experiments demonstrated that fibrinogen is transcriptionally downregulated in presence of ORF3.

<table>
<thead>
<tr>
<th></th>
<th>Aα</th>
<th>Bβ</th>
<th>γ</th>
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<tbody>
<tr>
<td>Plasmid</td>
<td>O3</td>
<td>V</td>
<td>O3</td>
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<tr>
<td>Densitometry units</td>
<td>23.8 ± 1.87</td>
<td>98.18 ± 2.56</td>
<td>16.48 ± 1.45</td>
</tr>
</tbody>
</table>

**Fig. 3.2.11. RT-PCR analysis of fibrinogen mRNA.** First strand cDNA was reverse transcribed from total RNA and amplified by PCR using specific primers for Aα (lanes 1 and 2), Bβ (lanes 3 and 4) and γ (lanes 5 and 6) chain mRNA (top panel) from both ORF3 (lanes 1, 3 and 5) and vector (lanes 2, 4 and 6) transfected cells. The bottom panel shows amplified β-actin cDNA from the corresponding samples. The amplified products were resolved by electrophoresis on 1% agarose gel.