270 V) into 5 x 10^6 IHH using a BioRad Gene pulse Xcell system (Hercules, CA). The transfected cells were plated on collagen coated plastic dishes, and maintained in culture for HCV replication. Total cellular RNA was extracted 5 days post transfection. For detection of HCV genome, total cellular RNA and random hexamer were used for cDNA synthesis with a SuperScriptIII first-strand synthesis system (Invitrogen), following supplier's protocol. PCR amplification was performed with cDNA as a template, using sense (5'-ACCCGCTGAATTCCTGGAGA-3') and antisense (5'-CACGGTCTTCTAGACCTCCC-3') primers from 5'UTR, at 94°C for 30s, annealing at 55°C for 60s, extension at 72°C for 90s. GPDH was used as an internal control, using specific primers (17). RT-PCR analyses suggested amplification of 120 bp sequence from the 5'UTR (Fig. 1, panel A). In contrast, cells transfected with H77/GND RNA did not exhibit the presence of HCV genomic sequence. To rule out the integration of H77 plasmid DNA into IHH, the genomic DNA from cell lines were isolated and examined for HCV genome by PCR. Our results suggested the absence of HCV sequence, indicating HCV genomic RNA replication in the cytoplasm of IHH (data not shown). Filtered culture supernatant was also treated with RNaseA prior to isolation of viral RNA. RT-PCR was performed for NS5A region (17) and we have observed amplification of specific RNA sequence.

Western blot analysis was performed to analyze the expression of core and NS3 proteins in control and experimental cells using specific antibodies. An equal amount of proteins from whole cell lysates in sample buffer were separated by SDS-PAGE. Proteins were transferred onto nitrocellulose, incubated with specific antibodies, and detected by chemiluminescence (Amersham, Piscataway, NJ). HCV core protein was detected by a specific rabbit antiserum, and NS3 was detected by a specific mouse monoclonal antibody (Virogen,Watertown, MA). Blots

were stripped and reprobed using a mouse monoclonal antibody to actin (Oncogene Science, Cambridge, MA). IHH supporting HCV genome replication displayed the presence of core (~21 kDa) and NS3 (~63 kDa) proteins (Fig. 1, panels B and C). On the other hand, IHH transfected with H77/GND RNA did not show a detectable level of core or NS3 proteins. A weak level of core protein was detected in this set of IHH for immortalization by HCV core protein (Fig. 1, panel B). IHH transfected with HCV full-length RNA were passaged at 4 or 5 day intervals. HCV RNA and protein expression were detected up to 12 days of cell culture, and discontinued for the lack of growth after 2 weeks.

To further examine intracellular expression of HCV protein, IHH transfected with H77 RNA were fixed with 3.7% formaldehyde and incubated at room temperature for 1h with monoclonal antibodies to NS5a (Biogenesis, Kingstone, NH). Cells were washed three times with PBS and stained with anti-mouse Ig conjugated with Alexa 568 (Molecular Probes, Eugene, OR), and mounted for fluorescence microscopy. Primary antibodies and secondary antibody-fluorochrome conjugates were titrated for use of optimum dilutions where there was no background fluorescence. We have observed cytoplasmic expression of NS5a (Fig. 2, panel A) in 60% IHH after 5 days of transfection. HCV genotype 2a (clone JFH1) has been shown to grow in Huh-7 cells or its derivatives (5, 15, 29, 32). *In vitro* transcribed RNA from clone JFH1 was used for transfection of IHH to determine if the immortalized hepatocyte cell line supports HCV growth. Intracellular localization of NS3 protein from JFH1 RNA transfected IHH was detected by immunofluorescence (Fig. 2, panel C). We have also used Huh-7.5 cells transfected with JFH1 RNA as a positive control (29) and observed NS3 expression by indirect immunofluorescence (data not shown). On the other hand, IHH similarly transfected with RNA

from H77/GND or JFH1/GND clone did not display virus protein expression by immunofluorescence (Fig. 2, panels B and D).

Immunogold localization of virus-like particles. Phase contrast microscopy suggested that HCV genome transfected IHH were swollen with large vacuoles in the cytoplasm, whereas negative controls did not show any detectable changes. We also examined for cellular changes using electron microscopy, and at the ultrastructural level some of these vacuoles appeared to be empty (Fig. 3, panels A and E). Others contained lipid (Fig. 3, panel E) or material isolated for degradation (Fig. 3, panel D). Ultrastructural changes also included an increased polymorphism of nuclei (Fig. 3, panel E). Immunogold labeling was performed for localization of HCV like particles in transfected IHH. For this, transfected IHH (4 days in culture) were detached from collagen coated petridishes by a brief trypsin treatment, pelleted in a microcentrifuge and fixed in 4% paraformaldehyde and 1% glutaraldehyde in PBS for 16h at 4°C. Following washing with PBS, cells were washed in distilled water, dehydrated in ethanol, and infiltrated with L.R.White resin (London Resin Company, Berkshire, UK). The cell pellets were polymerized in BEEM capsules (Ted Pella, Inc., Redding, CA) at -20°C under ultraviolet light. Thin sections were cut from blocks, collected on formvar-coated nickel grids, and blocked with 1% fish gelatin and 1% BSA in PBS for 10 min. Sections were incubated for 2h in 1:100 dilution (titrated before hand for best results) of monoclonal antibody to E1 glycoprotein (305/C3) or normal mouse IgG in PBS containing 0.1% BSA, washed in PBS containing 0.1% BSA and incubated for 1h in Protein A-10 nm colloidal gold (CG) diluted 1:200 in PBS containing 0.1% BSA. After washing with PBS, the grids were fixed for 3 min in glutaraldehyde, washed in distilled water, stained with uranyl acetate and lead citrate and photographed with a JEOL 100 CX electron microscope. No clusters of CG particles were

observed in controls which consisted of staining with normal mouse IgG, omitting the primary antibody or staining mock-transfected IHH with the 305/C3 monoclonal antibody. Several hundred cells were evaluated in each case. Immunogold labeling with E1 specific monoclonal antibody demonstrated the presence of HCV-like particles and E1 protein in IHH. Numerous labeled virus-like particles were observed in the cytoplasm (Fig. 3, panels A and E) and near the plasma membrane (Fig. 3, panel C) of H77 RNA transfected IHH. The labeled particles were ~50 nm in diameter. Extensive labeling was also associated with the rough endoplasmic reticulum consistent with the synthesis of E1 viral protein (Fig. 3, panel B). In addition, we observed cytoplasmic phagic vacuoles which contained gold-labeled virus-like particles (Fig. 3, panel D).

Processing of cells into LR White resin for immunogold localization omits the conventional osmium tetroxide fixation step to preserve antigenicity but comes at the cost of reduced tissue contrast. In addition, the identification of virus particles by immunogold labeling at the ultrastructural level can be tricky. For this, we carried out a series of control experiments to insure labeling specificity. First, we observed clusters of CG on virus-like particles, and single CG particles in the endoplasmic reticulum in several independent anti-E1 labeling experiments. Second, H77/GND RNA transfected IHH (negative controls) showed no such clusters of CG in the cytoplasm or single CG particles localized along the endoplasmic reticulum or membranes (Fig. 3, panel F). Third, sections of HCV genome transfected IHH incubated with normal mouse IgG at similar IgG concentrations as used for the anti-E1 antibody did not result in any specific immunogold labeling. Fourth, omitting anti-E1 antibody did not result in any specific immunogold labeling. Finally, CG particles in the anti-E1 labeling

experiments were primarily confined to cells and were not observed to any degree in the spaces around cells, again suggesting the labeling was specific for E1 protein in cells. Thus, the appearance of virus-like particles in RNA transfected IHH indicated that HCV 1a replicate and assemble as virus particles.

Infection of IHH by HCV from culture medium. We next examined the presence of HCV in cell culture medium from IHH. After different days of transfection, culture medium was filtered through a 0.45 μm cellulose acetate membrane (Millipore, Bedford, MA), and concentrated to ~10 - 20 fold by Millipore ultrafiltration (100 kDa cut off) and used for detection of HCV genomic sequence by RT-PCR (Fig. 4, panel A). Presence of HCV 5'UTR was detected in culture medium from HCV genome transfected IHH, but not from polymerase defective HCV RNA transfected IHH. We obtained ~$1.1x10^8$ genome copies/ml of culture medium using real-time RT-PCR, as described recently (32). Culture supernatant collected up to 7 days suggested a peak of HCV genome copy number between 4 and 5 days after tarnsfection.

Next, we determined whether the culture medium contains infectious HCV. For this, culture medium was serially two fold diluted and inoculated into naïve IHH. Cells were incubated for 4h, washed and incubated with fresh medium for 3 days before analysis of ffu/ml by indirect immunofluorescence for NS5a (H77 clone) or NS3 (JFH1 clone) as recently described (32). Nuclear staining was performed using TO-PRO3-iodide (Molecular Probes) and mounted for confocal laser scanning microscopy (Bio-Rad, Model 1024). A representative figure displaying infection of IHH by H77 or JFH1 is shown (Fig. 4, panel B). The number of

fluorescing cells was counted and correlated with dilutions of cell culture medium for determination of ffu/ml. We observed an ~ $4.5x10^4$ - $1x10^5$ ffu/ml of the cell culture medium after 5 days of transfection from both H77 and JFH1 clones.

We transfected *in vitro* transcribed H77 or JFH1 RNA into IHH and isolated the RNA from transfected cells. Culture supernatant was also collected for isolation of RNA and determination of infectivity (ffu/ml). Real-Time PCR suggested maximal HCV RNA accumulation from H77 at the intracellular level on day 2, and declined on day 5 (Fig. 4, panel C). We have observed higher genome copy number and infectious virus titer at day 4. Similarly, JFH1 RNA transfected IHH supernatant displayed a peak genome copy number of 10^8 /ml, and infectivity of ~ $7x10^4$ ffu/ml on day 4.

A HCV infected patient serum (OP1843) displaying neutralizing activity against VSV/HCV pseudotype (19) was used in determining neutralization of cell culture grown HCV. Serum from a healthy volunteer was used as a negative control in HCV neutralization assay. A two fold serial dilutions of heat inactivated serum was incubated with ~100 fluorescent focus unit of HCV generated from H77 clone at 37^0C for 30 minutes. Virus-serum mixture was added on naïve IHH and incubated for 3 days. Neutralization of fluorescent focus unit was determined from the inhibition of NS5a protein expression by immunofluorescence. The results are shown as % inhibition of fluorescent focus unit (Fig. 4, panel D). An ~60% infectivity was inhibited upon prior incubation of HCV in culture medium with the patient serum at 1/50 dilution. Similar inhibition at different dilutions with 3 other HCV infected patient sera was observed. In contrast, sera from 4 healthy individuals did not inhibit infectivity

at 1/10 dilution. These results suggested that infectious HCV particles released in the culture medium are neutralized by specific antibodies.

HCV RNA is directly translated, and the precursor viral polypeptide is cleaved proteolytically to form individual proteins, and the replicase complex amplifies the RNA via a minus strand intermediate. Plus strand RNA progeny are packaged into virus particles and acquire their envelope probably by budding into the lumen of the endoplasmic reticulum. HCV particles are likely to be exported via the constitutive secretory pathway. Based on this working principle, we have shown in this report that IHH support HCV genome replication and protein expression from genotype 1a. Immunogold labeling using a monoclonal antibody demonstrated localization of HCV E1 glycoprotein in the rough endoplasmic reticulum, and the formation of virus-like particles. We transferred culture medium from HCV replicating cells into naïve IHH and HCV infection was detected by RT-PCR and indirect immunofluorescence. We have also observed JFH1 replication and virus growth in IHH. The infectious unit appeared to be similar with JFH1 grown in Huh-7 cells or its derivatives. JFH1 may replicate with a higher efficiency than H77 at the RNA level in Huh-7 cells or its derivatives. However, we focused to determine the generation of infectious HCV from H77 and JFH1 in IHH. In our experimental system, we have observed a similar level of viral genome copy of H77 or JFH1 RNA transfected culture supernatant as well as the fluorescence focus unit. We did not purify virus particles for negative staining due to the relatively low infectious unit in the culture medium. Three different groups of investigators have reported different densities of HCV2a particles. Zhong et al. (32) observed a peak infectivity from an apparent density of 1.105 gm/ml, Wakita et al. (29) observed a density of ~1.15 gm/ml, and

Lidenbach et al. (15) observed a broad distribution of virus infectivity over a range of 1.01 to 1.12 gm/ml. A similar finding suggesting a variation of buoyant density of cell culture grown HCV 2a between 1.06 and 1.16 gm/ml was reported by Cai et al. (5). HCV is known to associate with serum immunoglobulin and lipoproteins (24). We did not determine the density of cell grown HCV1a as the number of particles are at least 100 fold lower relative to the other commonly cell culture grown viruses, and for its known association with other serum proteins.

Recently, HCV production was reported from a HCV-ribozyme construct of genotype 1a (clone H77) in Huh-7 cells, although infectivity of virus was not determined (8). Virus genome replication and assembly are multi-step processes, and are influenced by the intracellular milieu. Inhibition of host cell growth and induction of cytokines, such as interferons, may have an impact for cure of virus replication (3). Our study supports the proof of concept for HCV replication and assembly of genotype 1a in IHH. To our knowledge, this is the first report describing the generation of cell culture grown HCV from genotype 1a. We speculate that cellular defense mechanism against HCV infection is attenuated or compromised in IHH. Further studies should help to unravel the specific mechanisms for growth of HCV in IHH, and to address important biological questions for the life-cycle of HCV. Studies are in progress to determine the factors influencing virus growth, such as serial passage for adaptation in IHH, mutations at specific sites of HCV genome, and selection of cell populations for attenuated protective mechanisms. We will also characterize the biophysical properties of cell culture grown HCV and infectivity in available animal models in the near future.

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Note: While our manuscript was under revision, Yi et al. (Proc. Natl. Acad. Sci., 103, 2310-2315, 2006), have reported the growth of H77-S (containing five adaptive mutations) in Huh-7.5 cells.

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FIGURE LEGENDS

Fig. 1. HCV RNA and protein expression in IHH. **Panel A:** RT-PCR analysis was performed using 5' untranslated region (5'UTR) specific primers from RNA isolated at day 5 from two different sets of IHH transfected with H77/GND RNA as a negative control (lanes 1 and 2) and H77 RNA (lanes 3 and 4). GPDH was amplified as an internal control. The sizes of the amplified bands were verified from the migration of a φX174-HaeIII digested DNA marker (not shown). **Panel B:** Western blot analysis for core protein expression in H77/GND RNA transfected (lane 1), and H77 RNA transfected (lane 2) IHH, using a specific antiserum. The blot was reprobed with antibody to actin for similar protein load in each lane. **Panel C:** Western blot analysis for NS3 protein expression in two different sets of IHH transfected with H77/GND RNA (lanes 1 and 2) and H77 RNA (lanes 3 and 4), using a specific monoclonal antibody. The blot was reprobed with antibody to actin for similar protein load in each lane. The molecular weight of the protein bands were verified from the migration of protein molecular weight markers (Cambrex, Rockland, ME).

Fig. 2. Intracellular expression of HCV proteins. IHH transfected with RNA from H77 clone (panel A) or H77/GND RNA transfected negative control (panel B) were treated with NS5a specific monoclonal antibody for detection of protein expression by intracellular immunofluorescence after 5 days of transfection. IHH were similarly transfeceted with RNA from JFH1 clone (panel C) and JFH1/GND RNA transfected negative control (panel D) for intracellular localization of NS3 using a specific monoclonal antibody. Green color indicates NS5a staining and red color for NS3 staining.

Fig. 3. Immunogold localization of HCV E1 protein and virus-like particles in IHH with a specific monoclonal antibody. **Panel A:** Localization of virus-like particles in the cytoplasm are indicated by arrows. **Panel B:** Localization of HCV E1 protein to the rough endoplasmic reticulum is marked by arrows. **Panel C:** Localization of virus-like particles in the cortical cytoplasm adjacent to the plasma membrane. **Panel D:** Localization of virus-like particles in a large phagic vacuole in the cytoplasm of IHH is shown by arrows. **Panel E:** Clusters of CG indicated by arrows show virus-like particles in IHH. The labeled particle indicated by an arrow and with an asterisk is shown at higher magnification in the inset. As observed by light microscopy, IHH contain cytoplasmic vacuoles and lipid droplets. **Panel F:** H77/GND RNA-transfected control section of IHH incubated with monoclonal antibody to E1 glycoprotein did not exhibit immunogold labeling. Other negative controls consisted of labeling with normal mouse IgG and omitting the primary antibody (not shown). The abbreviations used are: C – cytoplasm, M – mitochondrion, PM – plasma membrane, RER – rough endoplasmic reticulum, V – vacuole, LD – lipid droplet, PV – phagic vacuole. Magnification bars are 0.25 μm in panels A-F, and 0.1 μm in the inset in panel E.

Fig. 4. Presence of HCV in culture medium and infectivity of naïve IHH. **Panel A:** RT-PCR analysis was performed for detection of 5'UTR from culture medium of HCV RNA transfected IHH. Filtered culture medium from IHH transfected with H77/GND RNA (lane 1), full-length H77 RNA (lane 2), JFH1/GND (lane 3), full-length JFH1 RNA (lane 4) were analyzed for amplification of 5'UTR. HCV genome was amplified similarly from Huh-7 cells transfected with JFH1/GND RNA (lane 5) and full-length JFH1 RNA (lane 6). Cloned H77 DNA was included as a positive control in PCR amplification (lane 7).

Panel B: Immunofluorescence of IHH at day 3 after infection with filtered culture medium from H77 (panel a) and JFH1 (panel b) for detection of NS5a and NS3 protein expression, respectively. **Panel C:** Generation of infectious HCV after transfection of H77 genomic RNA into IHH. *In vitro* transcribed H77 RNA (2ug) was electroporated into $1x10^6$ IHH. HCV RNA copies at the intracellular (• ▪▪ •) level and in the culture supernatant (numbers on top) were measured by real-time PCR at indicated days. Virus infectivity of the culture supernatant was determined in naïve IHH and is expressed as ffu/ml (black bars). **Panel D:** Neutralization of virus infectivity by HCV infected patient serum (black bars). Two fold serial dilutions of test serum was incubated with ~100 fluorescent focus unit of virus generated from H77 clone at 37^0C for 30 minutes. Virus-serum mixture was added on naïve IHH and incubated for 3 days for determination of fluorescent focus unit by indirect immunofluorescence using NS5a specific antibody. Similar experiment was performed in parallel with serum from a healthy individual (hatched bars). The results are presented as % inhibition of fluorescent focus unit, and variation from triplicate assays are indicated by error bars.

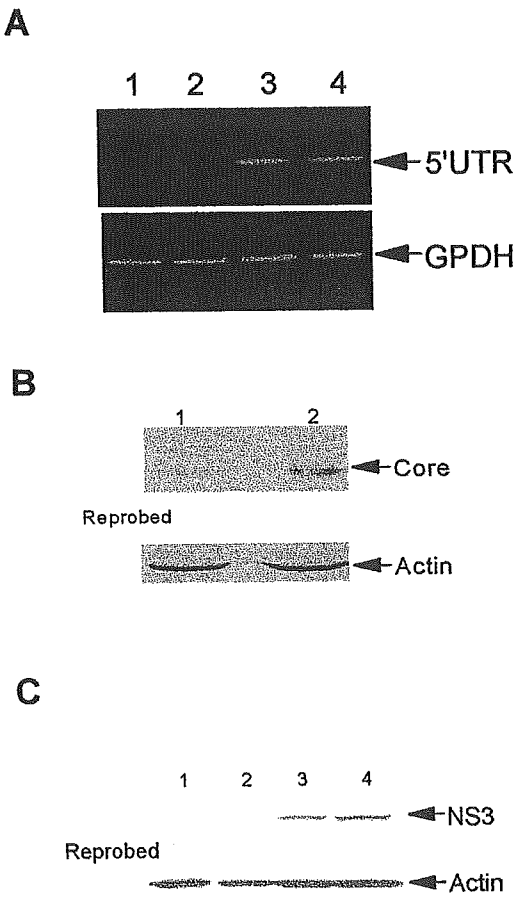
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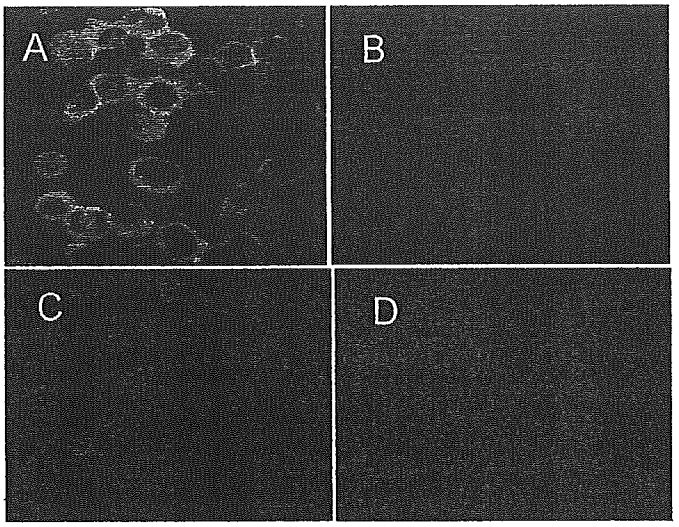
Fig. 1



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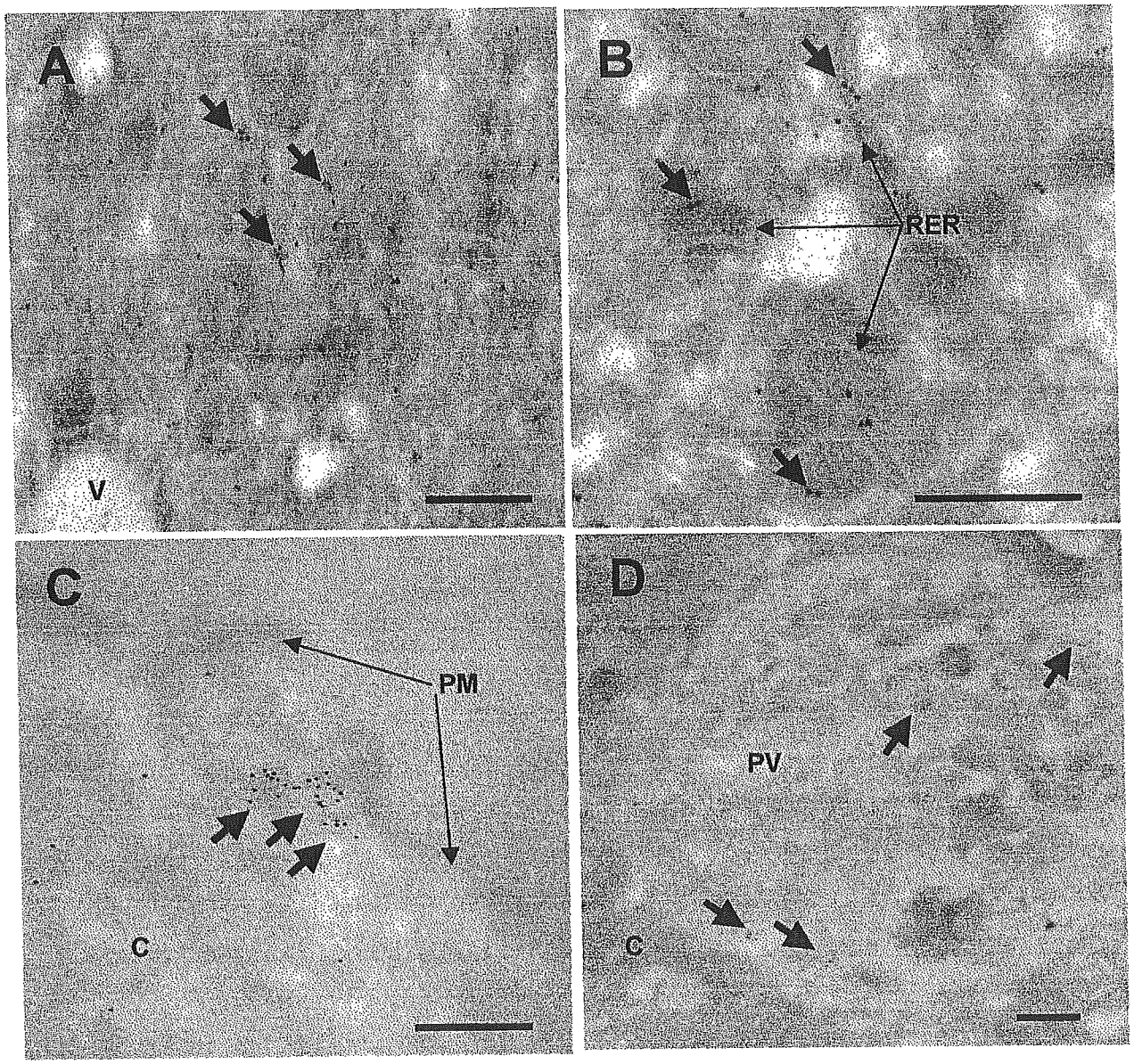

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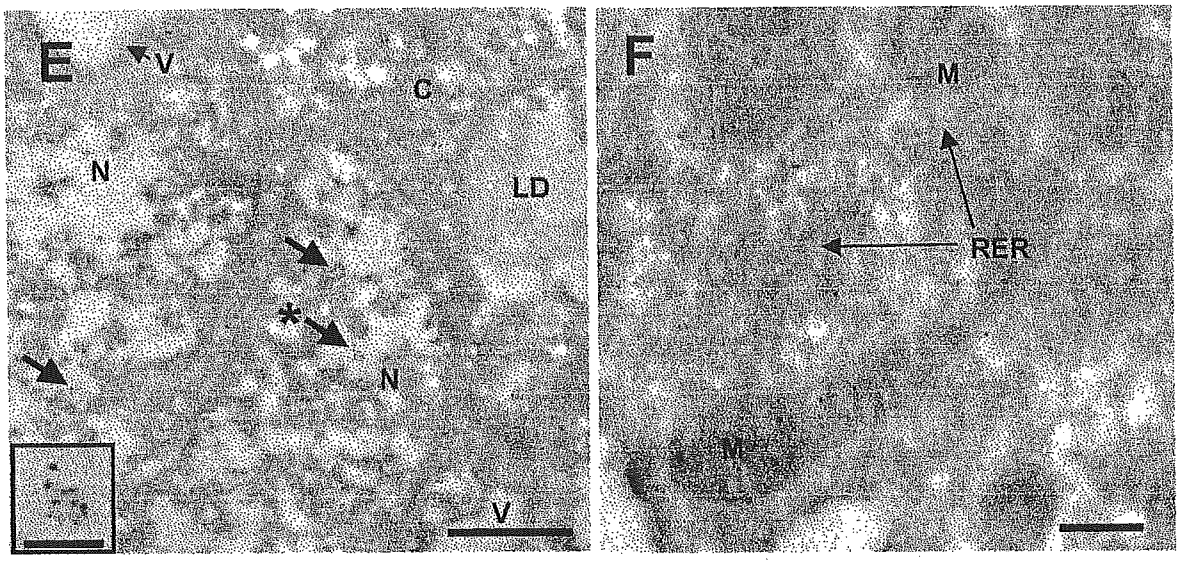

Fig. 3