The editosome for cytidine to uridine mRNA editing has a native complexity of 27S: identification of intracellular domains containing active and inactive editing factors

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Summary

Apolipoprotein B mRNA cytidine to uridine editing requires the assembly of a multiprotein editosome comprised minimally of the catalytic subunit, apolipoprotein B mRNA editing catalytic subunit 1 (APOBEC-1), and an RNA-binding protein, APOBEC-1 complementation factor (ACF). A rat homolog has been cloned with 93.5% identity to human ACF (huACF). Peptide-specific antibodies prepared against huACF immunoprecipitated a rat protein of similar mass as huACF bound to apolipoprotein B (apoB) RNA in UV cross-linking reactions, thereby providing evidence that the p66, mooring sequence-selective, RNA-binding protein identified previously in rat liver by UV cross-linking and implicated in editosome assembly is a functional homolog of huACF. The rat protein (p66/ACF) was distributed in both the nucleus and cytoplasm of rat primary hepatocytes. Within a thin section, a significant amount of total cellular p66/ACF was cytoplasmic, with a concentration at the outer surface of the endoplasmic reticulum. Native APOBEC-1 co-fractionated with p66/ACF in the cytoplasm as 60S complexes. In the nucleus, the biological site of apoB mRNA editing, native p66/ACF, was localized to heterochromatin and fractionated with APOBEC-1 as 27S editosomes. When apoB mRNA editing was stimulated in rat primary hepatocytes with ethanol or insulin, the abundance of p66/ACF in the nucleus markedly increased. It is proposed that the heterogeneity in size of complexes containing editing factors is functionally significant and reflects functionally engaged editosomes in the nucleus and an inactive cytoplasmic pool of factors.

Key words: APOBEC-1, Editing, Ethanol, Insulin, Nucleus

Introduction

Apolipoprotein B (apoB) mRNA editing involves a site-specific cytidine deamination reaction, which converts a CAA codon to a translation stop codon (Chen et al., 1987; Powell et al., 1987). Both apoB100 and apoB48 (translated from unedited and edited mRNA, respectively) are involved in dietary lipid transport. However, B100 lipoproteins are metabolized to atherogenic and cholesterol-rich low-density lipoproteins (LDL), whereas those containing B48 are not (Davidson, 1993; Chan et al., 1997; Acton et al., 1999).

Hepatic apoB lipoprotein synthesis, secretion and apoB mRNA editing are regulated during development, by hormones and by metabolic factors (Davidson, 1993; Chan et al., 1997; Smith et al., 1997). The cis-acting RNA sequence requirements for site-specific apoB mRNA editing have been delineated as a tripartite motif encompassing the edited cytidine at nucleotide 6666 (Smith, 1993; Sowden et al., 1998). The mooring sequence is the most 3′ element in the motif and is necessary and sufficient for editing. The minimal protein complement for editing in vitro in the human system consisted of ApoB mRNA editing catalytic subunit 1 (APOBEC-1), a 27 kDa cytidine deaminase responsible for base modification (Teng et al., 1993) and APOBEC-1 complementation factor (ACF), a 65 kDa protein with mooring sequence-selective RNA binding capacity (Mehta et al., 2000). APOBEC-1 has been proposed to bind to RNA at the editing site through its catalytic domain (Anant et al., 1995; MacGinnitie et al., 1995) and function as a head-to-tail homodimer (Lau et al., 1994; Oka et al., 1997; Navaratnam et al., 1998). APOBEC-1 alone cannot functionally interact with the editing site but rather acquires appropriate positioning at the editing site through its ability to bind to ACF, which in turn binds to the mooring sequence.

Human ASP (APOBEC-1 stimulating protein) was discovered in the same time frame as ACF and is identical to ACF, with the exception of an eight amino acid insertion at position 371 (Lellek et al., 2000). The ability of ASP to fully complement APOBEC-1 editing was dependent on its association with KSRP, previously identified as a KH-type splicing factor involved in alternative mRNA splicing of c-src (Min et al., 1997; Chou et al., 1999). The relative role(s) of ASP-KSRP-APOBEC-1 and ACF-APOBEC-1 as minimal editosomes in apoB mRNA editing is not understood. The simplicity suggested for the editosome assembled under

defined in vitro conditions contrasts significantly with the aggregate size of 27S (predicted to be ≥500 kDa) observed for editosomes assembled in extracts (Smith et al., 1991; Harris et al., 1993; Yang et al., 1997a). In this regard, multiple proteins have been proposed to interact with APOBEC-1 and modulate its editing activity (Lau et al., 1990; Harris et al., 1993; Navaratnam et al., 1993; Schock et al., 1996; Lau et al., 1997; Yang et al., 1997a; Greeve et al., 1998; Blanc et al., 2001). The composition and functional organization of the regulated apoB mRNA editosome therefore remains an open question.

ApoB mRNA editing occurred during or immediately after pre-mRNA splicing (Lau et al., 1991; Sowden et al., 1996) and metabolic stimulation of editing activity has been shown to occur in the nucleus (Yang et al., 2000). These data showed that apoB mRNA editing is a nuclear event. The intracellular distribution of APOBEC-1 and ACF have only been studied in transfected cells (Yang et al., 1997b; Yang et al., 2000; Blanc et al., 2001). These studies showed that recombinant APOBEC-1 and ACF were distributed in both the nucleus and cytoplasm of hepatoma cells. Editing normally does not occur in the cytoplasm. However, overexpression of APOBEC-1 in transfected cells induced editing of cytoplasmic apoB mRNA (Yang et al., 2000), and cytoplasmic extracts from normal hepatocytes supported apoB mRNA editing under the conditions of the in vitro assay (Harris et al., 1993). Taken together, the data suggested that the apoB mRNA editing factors are distributed in both the nucleus and cytoplasm of hepatocytes but that these populations may not be functionally or structurally equivalent, or both.

We report the molecular cloning of the rat homolog to ACF (p66/ACF) and show that it is p66, the mooring sequence-selective RNA-binding protein proposed to be involved in editing site recognition and editosome assembly (Harris et al., 1993; Navaratnam et al., 1993). Cytoplasmic p66/ACF was associated with the exterior surface of the endoplasmic reticulum and localized to heterochromatic regions in the nucleus. Sedimentation analysis revealed p66/ACF-APOBEC-1 as cytoplasmic 60S complexes and nuclear 27S editosomes. These studies suggest that the observed heterogeneity in editosome higher-order structure is subcellular-compartment specific and hence functionally significant.

Materials and Methods

Cells and culturing conditions

Rat primary hepatocytes were isolated from male Sprague-Dawley rats (200-250 g body weight) as described previously (Van Mater et al., 1998) and cultured in type I collagen-coated plastic dishes (Becton Dickinson, Bedford, MA) in Weymouth's 752/1 media (Sigma Chemical Co., St Louis, MO) lacking insulin. Ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY) or porcine insulin (Sigma) at final concentrations of 0.45% and 10 nM, respectively, were added 16-24 hours after plating cells, and cells were cultured for an additional 6 hours.

Molecular cloning of p66/ACF

A lambda gt11 rat liver cDNA library (Clontech, Palo Alto, CA) was screened under moderate stringency with random hexamer primed, radiolabeled full-length human ACF (huACF) cDNA. Two clones were identified after tertiary level screening that corresponded to the 5′ 600 bp and 3′ 650 bp of ACF. To isolate the middle portion of rat ACF, 3′ RACE was performed on Marathon rat liver cDNA (Clontech) according to the manufacturer's recommendations. Three identical 1.1 kb clones were isolated that overlapped both 5′ and 3′ lambda clones. The DNA sequence of rat ACF was verified by sequencing of RT-PCR products from rat liver poly A+ RNA. P66/ACF cDNA was subcloned into a modified *pcDNA*III vector such that it was expressed with a hexa-histidine motif and tagged with haemagglutinin (HA) and V5 epitopes at the 5′ and 3′ termini, respectively. The rat p66/ACF cDNA encodes 586 amino acids.

To produce recombinant protein, p66/ACF cDNA encoding a C-terminal 6His and HA-tag was subcloned into the yeast vector pYES 2.0 and under galactose-inducible control. Pelleted yeast were resuspended in an equal volume of 50 mM Tris, pH 8.0, 250 mM KCl, 10% glycerol, 5 mM $MgSO_4$, 2 mM β-mercaptoethanol, 1 mM PMSF, 5 μg aprotinin/ml (USB, Cleveland, OH), 5 μg leupeptin/ml (USB), 0.5 μg pepstatin (USB), 2 mM benzamidine and 1 g of alumina Type 305 powder (Sigma) per 5 ml yeast suspension added before freezing in liquid nitrogen.

Frozen yeast suspension was ground extensively in a porcelain mortar and pestle under liquid nitrogen, thawed and digested with 5 μg/ml DNase I (37°C, 20 minutes). Extracts were adjusted to 300 mM NaCl and cleared supernatants adsorbed to nickel affinity resin (Qiagen, Valencia, CA) according to the manufacturer's protocol. Adsorbed resins were washed sequentially with 1 M NaCl, 0.4% Triton X-100 and 50 mM Tris, pH 8.0, 50 mM NaCl, 10% glycerol and eluted in the later buffer containing 300 mM imidazole. P66/ACF was concentrated by dialysis against 20% polyethylene glycol (8,000 MW), and then dialyzed against and 50 mM Tris, pH 8.0, 50 mM NaCl, 10% glycerol and stored at –25°C until use.

Northern blot analysis

A Poly A+ northern blot (Clontech) was probed with a full-length p66/ACF cDNA radiolabeled with α ^{32}P[dCTP] using the RTS RadPrime DNA labeling system (GibcoBRL) according to the manufacturer's protocol. Blots were hybridized to probe (1×10^6 cpm/ml) in ExpressHyb (Clontech) and washed according to the manufacturer's recommendations.

Preparation of peptide-specific antibodies against ACF and immunoassays

An N-terminal domain in human ACF corresponding to residues 4-19 was identified by Mehta et al. (Mehta et al., 2000) as an accessible epitope for antibody production. This peptide was synthesized, analyzed by mass spectroscopy, conjugated to keyhole limpet haemocyanin and used to immunize rabbits (Bethyl Laboratories, Inc. Montgomery, TX). The resultant IgG was affinity purified.

Proteins from whole extracts or glycerol gradient fractions were resolved by SDS/10.5% PAGE, transferred to BA85 nitrocellulose membrane (Schleicher & Schuell, NH), probed with anti-ACF (1:5000 dilution) and a secondary peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA) (1:3000 dilution) as described previously (Yang et al., 1997a) and visualized by chemiluminescence (Renaissance, NEN Life Science Products Inc., Boston, MA). Alternatively, the primary antibody (200 μg) was adsorbed to 50 μl of packed Protein A-conjugated sepharose beads (Calbiochem, La Jolla, CA) and, following washes with 50 mM Tris, pH 8.0, 50 mM NaCl, used as an immunoprecipitation reagent (see below).

Immunofluorescence and immunohistochemistry

McArdle cells, grown on glass slides, were fixed with 2% paraformaldehyde, permeabilized with 0.4% Triton X100, blocked with 1% BSA and reacted with affinity-purified anti-ACF and affinity-purified FITC-conjugated goat anti-rabbit secondary antibody (Organon Teknika, West Chester, PA), each at 1:1000 dilution.

Slides were observed and photographed under an Olympus BH-2 fluorescence microscope using a 100× Olympus oil objective.

For immunohistochemistry, rat livers were perfused in situ with 20 ml of ice-cold 0.33 M STM (0.33 M sucrose, 50 mM Tris, pH 6.8, 5 mM $MgCl_2$ 10 mM sodium fluoride) containing 2 mM EGTA, 1 mM PMSF, 5 μg aprotinin/ml, 5 μg leupeptin/ml and 3,000 U of SUPERase-In RNase inhibitor (Ambion Inc., Austin, TX). Liver sections were fixed in 10% neutralized formalin (Fisher HealthCare, Swedesboro, NJ) for 6-8 hours followed by washes with PBS, paraffin embedding and cutting of 5 μm sections. Endogenous biotin and peroxidase were blocked in the sections using avidin (kit SP-2001, Vector Laboratories Burlingame, CA) and 3% H_2O_2, respectively. Sections were incubated in 1/100 anti-ACF overnight at 4°C, visualized by streptavidin-biotin technique (Grumbach and Veh, 1995) and counterstained with hematoxylin.

Immunoelectron microscopy

Rat livers were perfused as described above, diced into 5 mm cubes and fixed in phosphate-buffered, 4.0% paraformaldehyde for 6 hours, rinsed in Sorensen's phosphate buffer overnight, embedded into agarose and vibratomed into 50-80 μm sections. Sections were blocked overnight at 4°C as described by Brandstatter et al. (Brandstatter et al., 1997) and incubated with anti-ACF antibody (diluted 1:100 in PBS containing 1.0% normal goat or horse serum), 0.8% BSA and 0.1% fish gelatin at 4°C for 5 days. Sections were extensively rinsed in PBS at room temperature, incubated with a 1/200 dilution of a biotinylated secondary antibody and incubated overnight at 4°C. Sections were rinsed in PBS, reacted with a 1/300 dilution of extrAvidin (Sigma) developed with diaminobenzedine (DAB) and fixed in 2.0% gluteraldehyde (Brandstatter et al., 1997). DAB-labeled sections were silver enhanced, gold toned and post fixed in 1.0% osmium tetroxide (Brandstatter et al., 1997). Dehydrated sections were embedded in Spurr epoxy overnight, sectioned (80 nm), stained with uranyl acetate and lead citrate and examined using a Hitachi 7100 electron microscope.

Analysis of editing complexes, RNA-binding proteins and editing activity

S100 extracts were sedimented through 10%-50% glycerol gradients and the relative S value determined from the sedimentation of size markers in parallel gradients (Harris et al., 1993). Gradients were fractionated from the top, and an equal aliquot of each fraction subjected to western blot analysis and in vitro editing assays.

RNA-protein interactions were evaluated by UV cross-linking as described previously (Harris et al., 1993; Smith, 1998). Briefly, a 498 nt ^{32}P radiolabeled apoB RNA substrate was incubated with the 27S glycerol gradient fraction of rat liver nuclear S100 extracts to assemble editosomes. The reaction was exposed to 254 nm UV light for 5 minutes, RNase A and T_1 digested, resolved by SDS PAGE and the radiolabeled proteins visualized by autoradiography.

Alternatively, samples prepared by the UV cross-linking assay were incubated with 50 μl of anti-huACF-Protein A-sepharose beads on ice for 1 hour, washed with buffer containing 1 M NaCl followed by 0.4% Triton X100 and eluted into TriReagent (for RNA analysis) or 3 M sodium thiocyante and acetone precipitated (for protein analysis).

In vitro editing activity was determined in 100 μl reactions by incubating whole S100 extracts or glycerol gradient fractions with apoB RNA (Harris et al., 1993). In vivo editing activity was determined on total cellular RNA amplified by RT-PCR using primers specific for apoB sequences encompassing the editing site at C6666 (Van Mater et al., 1998; Yang et al., 2000). The proportion of unedited (CAA) and edited (UAA) RT-PCR products were determined by the poisoned primer extension assay and denaturing gel analysis (Smith et al., 1991).

Subcellular fractionation and extract preparation

Nuclei and cytoplasm were prepared from primary hepatocytes using NE-PER kit protocol (Pierce, Rockford, IL) and modifications as described recently (Yang et al., 2000). Proteinase, RNase and phosphatase inhibitors were added to all buffers as described for 0.33 M STM perfusion buffer. Eight 100 mm plates of rat primary hepatocytes, either with or without treatment with ethanol or insulin, were scraped into CER-I buffer. Nuclei were sedimented through 2.2 M sucrose containing STM, resuspended in 0.33 M STM and examined for purity by phase microscopy as described previously (Yang et al., 2000). All procedures were performed at 4-7°C.

Nuclei and cytoplasm were isolated from whole rat liver for S100 editing extract preparation as described previously (Smith et al., 1991), with the following modifications. Rat livers were perfused in situ as described above and nuclei were purified through 2.2 M sucrose in STM (Smith and Berezney, 1983). Nuclei were resuspended in extract buffer (EB, 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$ 10 mM sodium fluoride, 0.2 mM DTT, 2 mM EGTA, 1 mM PMSF, aprotinin (5 μg/ml), leupeptin (5 μg /ml), brought to 250 mM NaCl, homogenized and incubated on ice for 20 minutes. S100 nuclear extracts were obtained by microcentrifugation at 14,000 ***g*** for 20 minutes. S100 nuclear and cytoplasmic extracts were dialyzed against EB minus inhibitors for 4 hours at 7°C and stored at –20°C.

Results

Molecular cloning of the rat homolog to human ACF

The relationship between huACF (Mehta et al., 2000) and the previously demonstrated mooring sequence RNA-binding protein, p66 (Harris et al., 1993; Navaratnam et al., 1993), has not been addressed because huACF and p66 have been reported from heterologous systems and the cDNA encoding p66 was not available. To this end, a rat liver cDNA homologous to huACF was identified from a lambda gt11 rat liver cDNA library as described in Materials and Methods. The rat cDNA sequence was subcloned and aligned with huACF in Fig. 1. The amino acid sequence of p66 was 93.5% identical to that of huACF and equal to it in overall length. Most of the species-specific divergence in amino acid sequence occurred in the C-terminal half of these proteins. Relative to huACF, a glycine residue at position 327 was deleted but there was an alanine insertion at position 524. The proteins had identical sequence and positioning of the three RNA recognition motifs (RRMs) found in their N-termini. The eight amino acid insertion found in the homologous human protein ASP (Lellek et al., 2000) was not present in the rat sequence.

To confirm the functional relationship between rat p66 and huACF, a peptide-specific antibody was prepared against a conserved N-terminal 15 amino acid sequence (Fig. 1). Glycerol gradient fractions of the rat liver 27S editosome were reacted with radiolabeled apoB RNA under editosome assembly conditions and subjected to UV cross-linking. Subsequent to SDS PAGE and western transfer, membranes were autoradiographed to identify cross-linked proteins and then reacted with anti-p66/ACF antibodies. The result (Fig. 2) showed the well characterized p66 and p44 apoB RNA-binding proteins present in rat liver editosomes (Harris et al., 1993; Navaratnam et al., 1993; Yang et al., 1977b; Smith, 1998; Steinburg et al., 1999) and immunoreactivity with anti-p66/ACF to a single protein band of 66 kDa, migrating coincident with the center of the UV cross-linking signal (Fig. 2). The data supported the possibility that the mooring-

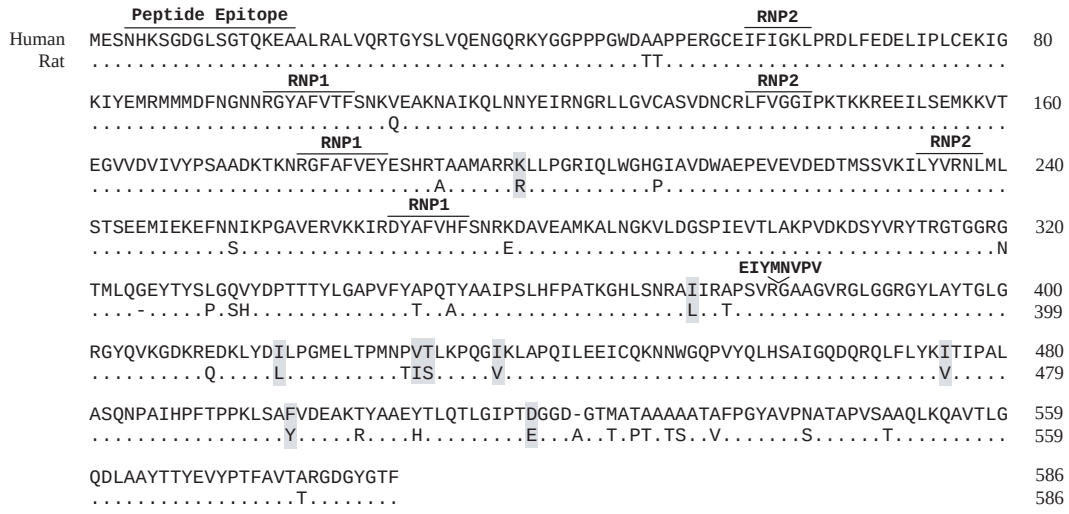


Fig. 1. Amino acid sequence of rat p66 and its alignment with huACF. P66 cDNA was amplified from total rat liver poly A mRNA. The predicted amino acid sequence has been aligned with that of huACF. For clarity, only those amino acids not identical to huACF are shown, with shading indicating a conservative substitution in the rat p66 sequence. The location of the RNP2 and RNP1 sequences of the three RRMs are indicated. The site of an eight amino acid insertion found in the human ASP homolog and the location of the 15 amino acid sequence used to produce peptide-specific antibodies are also indicated.

sequence-selective p66 RNA-binding protein is the rat homolog to huACF.

To validate that the immunoreactive rat liver protein was a homolog of huACF, in vitro editing reactions containing rat liver extract were evaluated by immunoprecipitation. Reactions containing either radiolabeled apoB RNA or a radiolabeled control RNA (WT-1, which lacked mooring sequences) (Yang et al., 1997a), were immunoprecipitated with the peptide-specific antibody against huACF and the amount of radiolabeled RNA recovered was quantified by liquid scintillation counting. Significant radiolabeled RNA only was immunoprecipitated from reactions containing radiolabeled apoB RNA (Fig. 3A). The yield of immunoprecipitated apoB RNA increased over the first 30 minutes of the reaction, consistent with the previously described kinetics of editosome assembly (Smith et al., 1991). The amount of immunoprecipitated WT-1 RNA remained low throughout the 60 minute reaction.

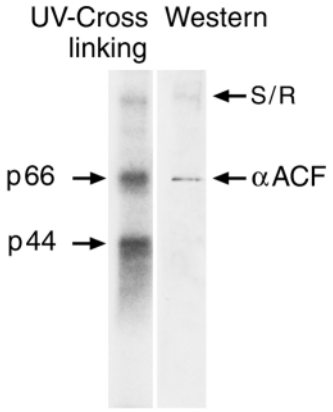


Fig. 2. Rat p66 mooring-sequence-selective, RNA-binding protein is a huACF homolog. Rat liver nuclear 27S editosome glycerol gradient fraction was UV cross-linked to radiolabeled apoB RNA. The proteins in the reaction were resolved by SDS PAGE, immunoblotted and exposed to X-ray film to determine the migration of the known 66 kDa and 44 kDa apoB RNA-binding proteins (left lane). Subsequently, the blot was reacted with anti-ACF (right lane). The positions of p66 and p44 UV cross-linked proteins and the cross-immunoreactive p66 are indicated. The stacking/running gel interface (S/R) is also indicated for reference.

The RNA-binding protein responsible for the selective recovery of apoB RNA in the immunoprecipitates was evaluated by PAGE and autoradiography (Fig. 3B). To demonstrate the effectiveness of the immunoprecipitation conditions, recombinant huACF was reacted with radiolabeled apoB RNA in the presence of 1000-fold molar excess of competing unlabeled WT-1 RNA or apoB RNA and subjected to immunoprecipitation. A radiolabeled band corresponding to huACF was observed under these conditions but was significantly reduced when unlabeled apoB RNA was used as competitor. Reactions containing rat liver nuclear extract, radiolabeled apoB RNA and 1000-fold molar excess of competing unlabeled WT-1 also yielded a radiolabeled protein of approximately 66 kDa. By contrast, when the reactions were carried out in the presence of excess cold apoB RNA, virtually no radiolabeled protein could be detected. RNA excess competition analyses have been used previously to demonstrate the selectivity of p66 UV cross-linking to apoB mRNA (Harris et al., 1993, Yang et al., 1997a). The current data demonstrated therefore that an apoB RNA-selective binding protein of the same size as huACF and cross-reactive with anti-huACF antibodies was present in rat liver extracts. Taken together, these data strongly suggested that p66 is the rat homolog of huACF. The rat homolog will be referred to as p66/ACF (Genbank accession number, AF290984).

P66/ACF mRNA and protein expression

Northern blot and RT-PCR analyses showed that huACF was expressed predominantly in liver and to a lesser extent in other tissues (Mehta et al., 2000). Northern blot analysis of rat poly A+ mRNA showed p66/ACF expression in liver and kidney as 3.0 kb and 9.5 kb transcripts (Fig. 4A). Expression of the smaller transcript was also observed in spleen, lung and testis. Probing of the same blot with GAPDH revealed approximate equal loading of RNAs from all tissues.

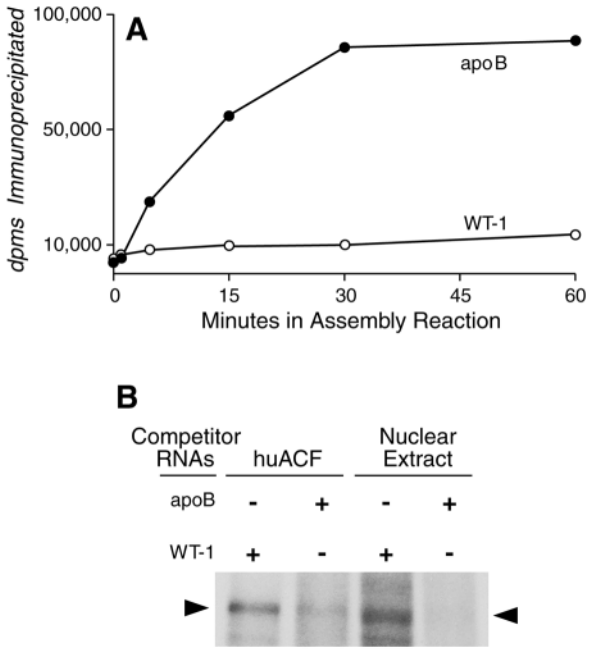


Fig. 3. Anti-huACF immunoprecipitation of rat p66 cross-linked to apoB RNA. (A) In vitro editing reactions containing rat liver nuclear extract and either radiolabeled apoB or control RNA (WT-1) were immunoprecipitated with anti-huACF at varying times during a 60 minute reaction and without RNase digest, subjected to immunoprecipitation with anti-huACF antibody bound to Protein A beads. The amount of RNA recovered in the immunoprecipitate was determined by scintillation counting washed beads. (B) In vitro editing reactions containing recombinant huACF or rat liver nuclear extract and radiolabeled apoB RNA, together with 1,000-fold molar excess of either WT-1 or apoB RNA (as indicated above each lane) were UV cross-linked after 60 minutes of reaction, RNase digested and then subjected to immunoprecipitation with anti-huACF antibodies. Immunoprecipitates were resolved on 10% SDS PAGE and autoradiographed.

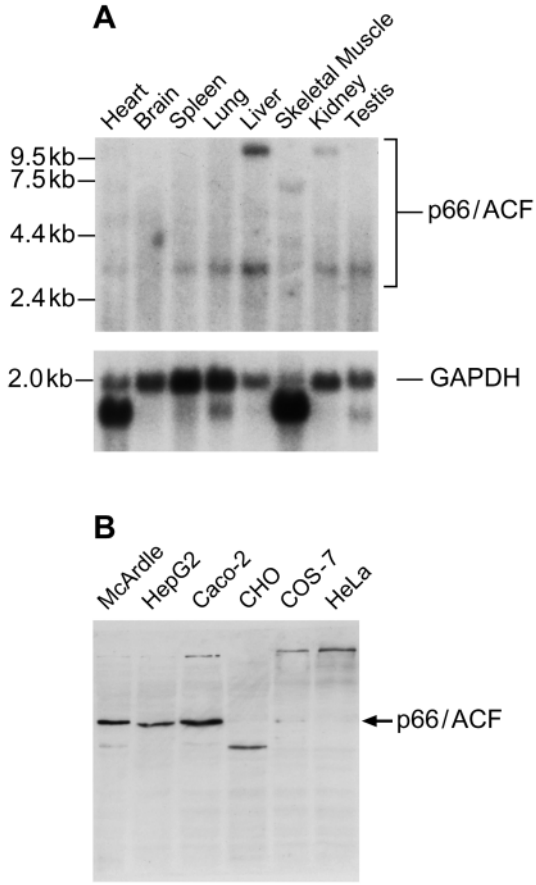


Fig. 4. Expression of P66/ACF mRNA and protein. (A) A northern blot of total cellular rat polyA+ mRNA from the indicated tissues was probed with p66/ACF cDNA and autoradiographed. The relative migration of size markers is shown to the left. (B) A western blot of total cellular protein from the indicated cell lines was reacted with peptide-specific polyclonal antibodies and visualized by chemiluminescence and autoradiography.

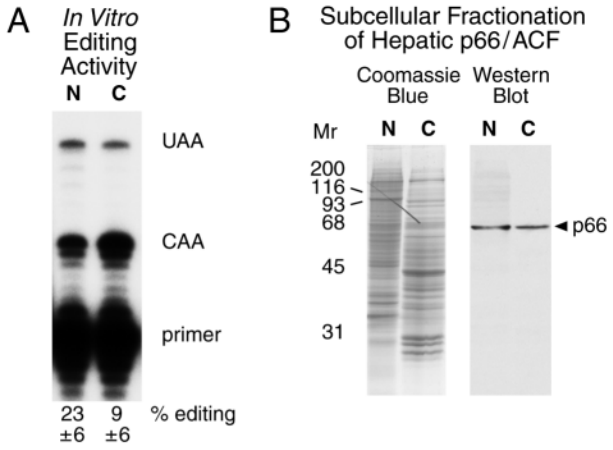
Protein-expression levels were evaluated in cell lines commonly used to study apoB mRNA editing. P66/ACF was prominent in McArdle rat hepatoma cells, HepG2 human hepatoma cells and Caco-2 human colorectal carcinoma cells (Fig. 4B). Far less p66/ACF was detectable in monkey kidney cells (COS-7). Chinese hamster ovary cells (CHO) expressed a cross-reacting protein of approximately 52 kDa but p66/ACF expression was below detection limits in human cervical carcinoma cells (HeLa).

P66/ACF is in nuclear and cytoplasmic liver extracts

Editing activity has been studied in this laboratory using liver homogenate, post-nuclear S100 extracts. Given the preponderance of evidence supporting the nuclear localization of editing activity (Lau et al., 1990; Lau et al., 1991; Sowden et al., 1996; Yang et al., 2000), we evaluated editing activity and the organization of editing factors in S100 extracts from both rat liver cytoplasm and from purified nuclei. On a per microgram protein basis, nuclear extracts were more than twice as efficient in editing exogenous apoB RNA substrates than cytoplasmic extracts in the standard in vitro editing assay (Fig. 5A). Western blots containing identical amounts of nuclear and cytoplasmic proteins that were reacted with anti-ACF showed p66/ACF in both fractions (Fig. 5B). Whole-band, densitometric quantification showed that there was 1.5-fold higher p66/ACF immunoreactivity in the nuclear lane compared to that in the cytoplasmic protein lane. However, correcting this ratio for the total amount of protein recovered in each fraction relative to that loaded on the gel (see Materials and Methods) revealed that, on average, 96% of the total cellular p66/ACF was recovered in the cytoplasmic S100 extract (Fig. 5C).

Immunolocalization of p66/ACF

We recently showed that exogenously expressed huAPOBEC-1 and huACF were distributed in the nucleus and cytoplasm of transfected McArdle rat hepatoma cells (Yang et al., 1997b; Yang et al., 2000). As these cells expressed sufficient endogenous p66/ACF for western blot detection it was possible to re-evaluate the intracellular distribution of p66/ACF suggested by biochemical fractionation in the context of intact cells. Indirect immunofluorescence using the peptide-specific antibody showed homogeneous nuclear p66/ACF reactivity in



C p66/ACF Recovery in Liver Cell Subfractions

	Relative Immunoreactivity per μg protein	Total Protein per fraction (mg)	Total p66/ACF ($x10^{-3}$)	Relative % p66/ACF per fraction
Nuclear Extract	1.5	42	63	4
Cytoplasmic Extract	1	1365	1365	96

Fig. 5. Recovery of p66/ACF during rat liver nuclear and cytoplasmic fractionation. (A) Normal rat liver nuclear and cytoplasmic S100 extracts were prepared and subjected to an in vitro editing reaction and poisoned primer extension quantification of editing. RNA editing was quantified by PhosphorImager densitometry as the number of counts in edited RNA (UAA) divided by the sum of the counts in UAA and the unedited RNA (CAA) times 100. The reactions were performed in triplicate and shown with the s.e.m. (B) 25 μg of protein from nuclear (N) and cytoplasmic (C) S100 extracts were resolved by SDS PAGE and stained with Coomassie blue (left pair of lanes) or western blotted with anti-ACF as described in Materials and Methods (right pair of lanes). The migration of molecular mass marker proteins is shown to the left (Mr). (C) Chemiluminescence from western blots in B were quantified, setting the density in the cytoplasmic lane to an arbitrary unit of 1 (first column). The total amount of protein in each fraction was calculated (second column) and this value was multiplied by the relevant number in column 1 to give the estimated amount of total p66/ACF in each fraction (column three). The relative percent p66/ACF in each fractions (column four) was calculated assuming that the sum of the nuclear and cytoplasmic p66/ACF equalled the total tissue p66/ACF. On average, 35 ml of cytoplasmic S100 extract at 39 mg protein/ml and 2 ml of nuclear S100 extract at 21 mg protein/ml were obtained.

McArdle cells together with cytoplasmic staining in the form of a highly reactive array of aggregates (speckling) varying in size (Fig. 6).

McArdle cells are an immortal cell line and therefore the distribution of proteins may not reflect that in normal hepatocytes. The histological distribution of p66/ACF was evaluated using formalin-fixed, rat liver sections. These did not reveal uniform staining, but rather, zones of hepatocytes were more intensely immunoreactive than others (Fig. 7). These may correspond to hepatocytes known to be actively synthesizing and secreting lipoproteins localized around the central veins that drain the liver (Tanikawa, 1979).

The ultrastructural distribution of normal hepatocyte p66/ACF was evaluated by immunoelectron microscopy. P66/ACF immunoreactivity was observed in both the cytoplasm and nucleus of in situ hepatocytes (Fig. 8A). Nuclear p66ACF was observed associated with heterochromatin (Fig. 8B; Fig. 9A,B). In peripheral heterochromatin (the chromatin associated with the nuclear lamina), p66/ACF tended to localize at the borders of heterochromatin and the interchromatin regions. The interchromatin domain itself and the nucleolus contained low or no immunoreactivity.

Cytoplasmic p66/ACF immunoreactivity was concentrated along regions of endoplasmic reticulum (Fig. 8A), predominantly at the outer surface of the endoplasmic reticulum membrane (Fig. 8B; Fig. 9A). The lumen of the endoplasmic reticulum had low or no immunoreactivity. Some immunoreactivity was observed along the surface of the Golgi apparatus (Fig. 9B) but mitochondria had no or trace reactivity (Figs 8, 9).

P66/ACF in 27S and 60S complexes

The finding that p66/ACF immunolocalized to both heterochromatin regions and on the outer surface of the ER raised the question as to whether there were distinguishing physical characteristics underlying its distribution. To evaluate the aggregate size of complexes containing p66/ACF, and specifically to determine whether 27S editosomes exist in rat liver, cytoplasmic and nuclear extracts were prepared and resolved by glycerol gradient sedimentation without prior in vitro incubations. As a control for both the selectivity of the nuclear and cytoplasmic fractionation and possibility of aggregation of RNA-protein complexes during extract preparation or sedimentation, blots from the cytoplasmic and nuclear gradient fractions (Fig. 10A and 10B, respectively) were reacted with antibodies specific for KSRP, a protein involved in alternative pre-mRNA splicing. Significant immunoreactivity was only detected in nuclear fractions 1-4 with a peak in fraction 2 or 8S (Fig. 10B), suggesting that leaching of nuclear proteins and aggregation of RNA-binding proteins had not occurred.

Cytoplasmic p66/ACF immunoreactivity sedimented as heterogeneous complexes recovered in fractions 3-11 corresponding to 10S to ≥100S (Fig. 10C). Native APOBEC-1 was expressed below the detection limit of antibodies currently available, and therefore its presence could only be inferred from the ability of fractions to support editing in vitro. Cytoplasmic APOBEC-1 was not active in editing apoB mRNA within intact cells (Yang et al., 2000). However, it has been shown that the conditions of the in vitro editing assay could disaggregate and activate these complexes (Harris et al., 1993). Using these methods, cytoplasmic APOBEC-1 could be demonstrated to co-sediment with p66/ACF in fractions 5-10 with a peak of activity in fractions 7-9, corresponding to 60S (Fig. 10E).

Nuclear p66/ACF also sedimented in heterogeneous complexes but in contrast to cytoplasmic extracts, nuclear p66/ACF immunoreactivity was recovered in fractions 1-8, corresponding to 4S-60S (Fig. 10D). Nuclear APOBEC-1 co-sedimented with p66/ACF in fractions 3-9 with a peak in fractions 4-6 centered on 27S (Fig. 10F). This size is consistent

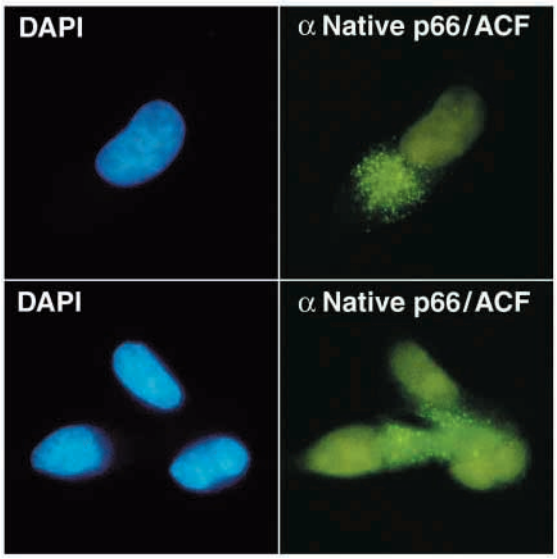


Fig. 6. Native p66/ACF is distributed in both the nucleus and cytoplasm of McArdle rat hepatoma cells. McArdle cells were grown on glass slides and prepared for immunofluorescence microscopy using anti-ACF. Two different fields are shown, with DAPI nuclear staining (left) and FITC fluorescence of anti-ACF reactivity (right).

with that characterized previously for the in vitro assembled functional editosome (Smith et al., 1991; Harris et al., 1993). Although strong p66/ACF immunoreactivity was also observed in the 8S-11S region of this gradient (Fig. 10D), little or no editing activity could be detected in these fractions (Fig. 10F), suggesting that this region was devoid of functional APOBEC-1. A low level of editing in complexes as large as 60S (Fig. 10F) suggested that APOBEC-1-p66/ACF complexes similar in size to those found in the cytoplasm (Fig. 10E) may be present as a minor component in the nucleus. The data argue strongly that the size of the minimal functional editosome (p66/ACF-APOBEC-1 complexes) in hepatocytes is 27S. Moreover, the data show that the previously identified 60S complex of editing factors in rat liver extracts is predominantly a cytoplasmic form, and therefore not functionally engaged in editing.

Induction of editing activity increased the amount of nuclear p66/ACF

The importance of the cell nucleus as the site of apoB mRNA editing led us to consider whether the amount of p66/ACF in the nucleus might have to change in response to metabolic conditions that stimulate editing activity. To evaluate this hypothesis, insulin and ethanol were selected as agents known to stimulate apoB mRNA editing activity. Insulin promoted enhanced editing activity by inducing the expression of APOBEC-1 through transcription and translation (Funahashi et al., 1995; Thorngate et al., 1994; Phung et al., 1996; von Wrongski et al., 1998). Ethanol also stimulated hepatic apoB mRNA editing but without inducing *apobec-1* gene expression (Lau et al., 1995; Van Mater et al., 1998).

Following 6 hours of treatment, with either ethanol or insulin, an equivalent amount of cytoplasmic and nuclear

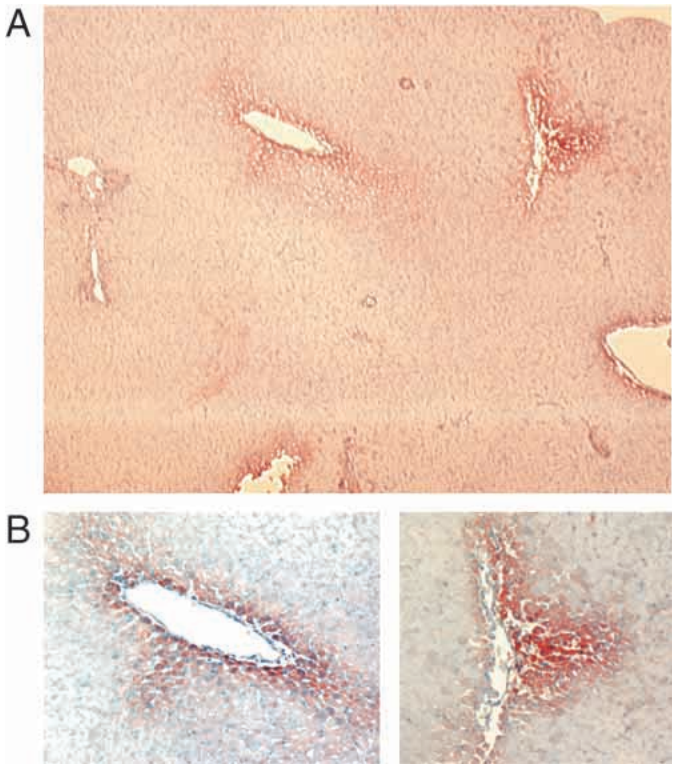


Fig. 7. Immunohistochemical localization of rat liver p66/ACF. Rat liver sections were prepared and subjected to immunocytochemistry using anti-p66/ACF. (A) Low power (20×) image of a liver section showing the distribution of central veins with associated dense p66 reactivity. (B) Higher power (40×) images of two of the central veins regions in A showing regionally high concentrations of p66/ACF reactivity. Individual hepatocytes were made apparent by nuclear staining with hematoxylin counter staining.

proteins from control, ethanol-treated or insulin-treated rat primary hepatocytes were resolved by SDS PAGE and western blots reacted with anti-p66/ACF antibodies. RNA was also isolated from cultures treated in parallel for the analysis of apoB mRNA editing. As predicted, ethanol and insulin treatment stimulated apoB mRNA editing from control levels 53%±3% s.e.m. to 86%±4% s.e.m. and 75%±3% s.e.m., respectively (Fig. 11). Whole band densitometric quantification of the p66/ACF signals from western blots showed that there was a marked increase in the proportion of immunoreactivity in the nucleus relative to the cytoplasm following ethanol and insulin stimulation, although the calculated mass of total liver p66/ACF from densitometric quantification (as described in Materials and Methods) did not change. The ratio of nuclear to cytoplasmic immunoreactivity (N/C ratio) increased from 1.7 in control hepatocytes to 7 and 10 in ethanol- and insulin-treated cells, respectively (Fig. 11), corresponding to an increase in the proportion of total cellular p66/ACF in the nucleus from 4% in control liver to 23%±6% s.e.m. and 31%±4% s.e.m. (n=3), respectively. These data are consistent with a regulatory mechanism whereby p66/ACF-APOBEC-1 complexes may be rate limiting and therefore an increase in p66/ACF's abundance in the nucleus (by import or retention) may facilitate metabolic stimulation of apoB mRNA editing activity.

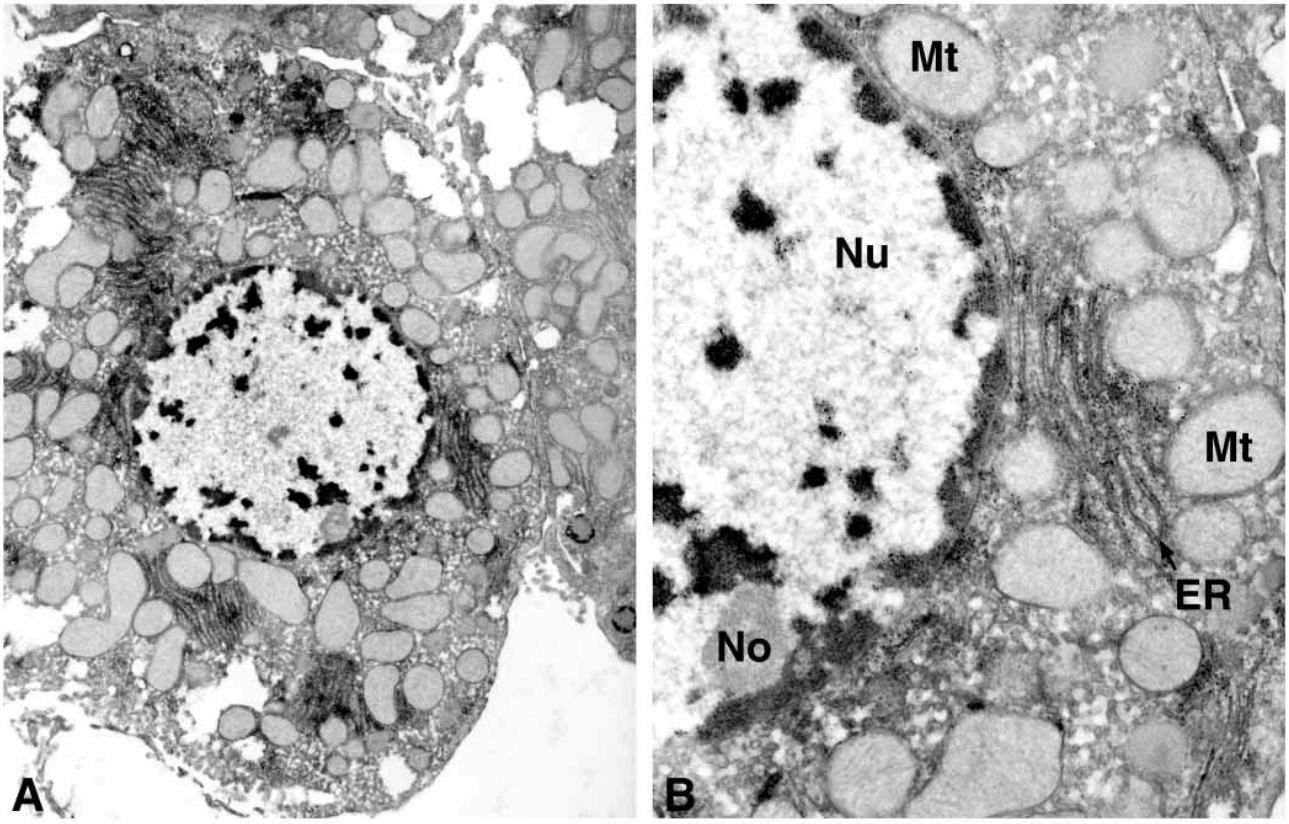


Fig. 8. Localization of rat liver p66/ACF by immunoelectron microscopy. Rat liver sections were prepared, reacted with anti-p66/ACF and detected with biotin secondary antibody and peroxidase conjugated extrAvidin, followed by enhancement with silver and gold toning. (A) Magnification, 7500×. (B) High magnification (22,500×) of the 3 o'clock area of the nucleus in A. ER, endoplasmic reticulum, Mt, mitochondria; No, nucleolus; Nu, nucleus.

Discussion

Molecular cloning of p66/ACF

This study reports on the cloning and sequencing of the rat homolog p66/ACF of human ACF, the auxiliary protein that is sufficient for in vitro complementation of APOBEC-1 in apoB mRNA editing. P66/ACF has been conserved between rats and humans, diverging only 6.5% in amino acid sequence identity. Evidence has been provided that p66/ACF and huACF are the 66 kDa RNA-binding proteins identified several years ago (Harris et al., 1993; Navaratnam et al., 1993) as a component of editosomal complexes whose mooring sequence selective interactions led to the in vitro assembly of functional 27S editosomes (Harris et al., 1993).

The subcellular distribution of native p66/ACF and APOBEC-1

To date, the expression of factors involved in apoB mRNA editing has been inferred by RT-PCR of mRNA encoding APOBEC-1 (Teng et al., 1993; Funahashi et al., 1995; Thorngate et al., 1994; Phung et al., 1996; von Wrongski et al., 1998), by UV cross-linking of auxiliary proteins in extracts to apoB RNA (Harris et al., 1993; Navaratnam et al., 1993; Yang et al., 1997a; Lellek et al., 2000) and the induction of editing activity following expression of recombinant factors in transfected cells (Yang et al., 1997b; Siddiqui et al., 1999; Yang et al., 2000; Yang et al., 2001; Blanc et al., 2001). In tissues where editing naturally occurs, APOBEC-1 was not expressed to high enough levels for detection with the currently available antibodies. The cellular localization of native APOBEC-1 and other editing factors in various tissues has therefore remained unknown.

It was somewhat surprising to find that recombinant APOBEC-1 and huACF (Yang et al., 1997b; Yang et al., 2000) were distributed in both the cytoplasm and nucleus when they were expressed in transfected cells. Overexpression of proteins is subject to the criticism that cellular transport mechanisms could become saturated and this could lead to artifacts in their intracellular distribution. This study has established that native p66/ACF was distributed in the nucleus and the cytoplasm of rat primary hepatocytes in situ. By virtue of the colocalization of editing activity, the data also suggested that some or all of native APOBEC-1 was also distributed with p66/ACF in the nucleus and cytoplasm. Immunoelectronmicroscopy further showed that p66/ACF was concentrated with or near heterochromatin in the nucleus and as regions of concentration on the surface of the endoplasmic reticulum.

Only a fraction of the total cellular p66/ACF is in the nucleus

The size of the apoB mRNA editosome has been suggested to be 27S, based on complexes assembled in vitro in extracts on

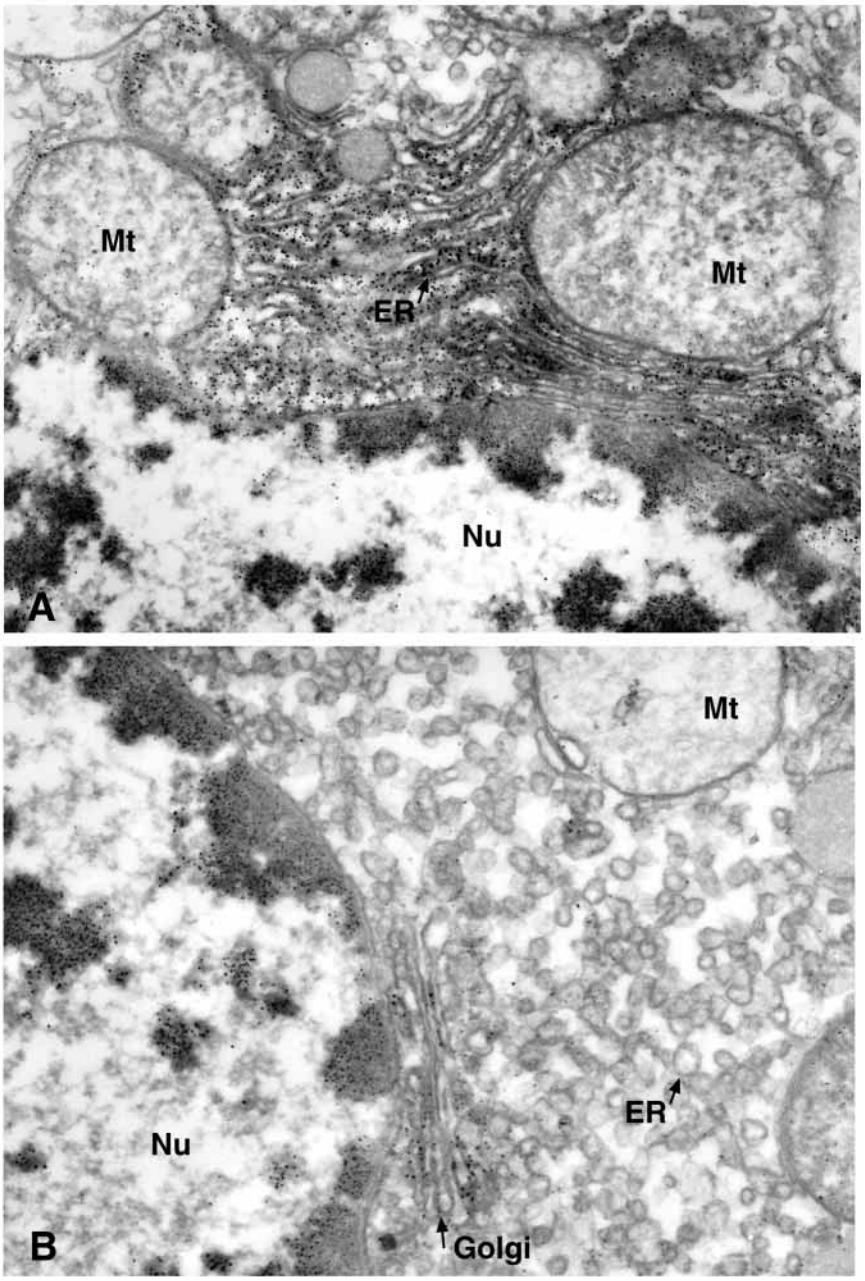


Fig. 9. Ultrastructural localization of p66/ACF. Sections of rat liver were prepared and reacted with p66/ACF as described in Fig. 8. (A) P66/ACF was associated with the surface and borders of heterochromatin in the nucleus (magnification, 22,500×). Cytoplasmic p66/ACF was predominantly associated with the outer surface of the endoplasmic reticulum and B, to a lesser extent with the outer surface of the Golgi (magnification, 30,000×). ER, endoplasmic reticulum; Mt, mitochondria; Nu, nucleus.

apoB reporter RNAs (Smith et al., 1991; Harris et al., 1993). This study has proven that 27S complexes of native p66/ACF and APOBEC-1 (the minimal functional components of editosomes) were the predominant form of biologically active, nuclear editosomes in rat primary hepatocytes. Support for the specificity of these complexes comes from the finding that although the sedimentation profile of p66/ACF was broad, APOBEC-1 and p66/ACF functional interactions were only observed within a discrete size distribution.

In addition to APOBEC-1 and p66/ACF there are likely to be other protein components that contribute to the estimated mass of ≥1.5 mDa of the 27S editosome. The alternative splicing factor KSRP was evaluated as a control for the potential loss of nuclear proteins to the cytoplasm during subcellular fractionation. However, sedimentation analysis suggested that a small fraction of native KSRP co-sedimented with 27S editosomes. This suggested the possibility that ASP may also be present in 27S complexes. However, formal proof of this possibility awaits the development of antibodies capable of distinguishing ACF from ASP.

RNA-binding proteins p100, p55 and p44 and αI3 serum proteinase inhibitor are candidate components of the 27S editosomes based on their co-purification with 6His-tagged APOBEC-1 overexpressed in McArdle rat hepatoma cells and affinity purified from cell extracts as functional editing complexes (Yang et al., 1997a). GRYRBP (Blanc et al., 2001; Lau et al., 2001), hnRNP proteins C (Greeve et al., 1998) and ABBP1 (Lau et al., 1997) are also candidate components of the 27S editosome: they all bind APOBEC-1, are nuclear proteins and modulate editing activity. The biological role of all of the proteins reported to associate directly or indirectly with APOBEC-1 remains to be shown. It is possible that there is heterogeneity in the composition of the 27S editosome that results from the regulation of its interaction with apoB mRNA and its catalytic activity.

Editing of ApoB mRNA may take place in association with heterochromatin domains

Given the obligate nature of p66/ACF in apoB mRNA editing, our data suggest that editosome assembly and perhaps editing might take place at the borders of heterochromatin and the interchromatin domain, known to be the sites of nascent transcript synthesis and ribonucleoprotein particle assembly (Puvion and Moyne, 1978; Fakan and Puvion, 1980; Shopland and Lawrence, 2000). Consistent with this possibility are data showing that apoB mRNA editing may occur on some transcripts prior to polyadenylation and that the majority of editing occurred coincident with or immediately after pre-mRNA splicing (Lau et al., 1991).

It is important to interpret these data cautiously, as we do not know whether the p66/ACF epitope is equally accessible throughout the nucleus (i.e. it may not be available to react with the antibody in the interchromatin domain). Moreover, studies of pre-mRNA splicing factors have shown them to be capable of intranuclear redistribution within cycles of splicing activity

and storage (Misteli et al., 1997; Kauffman and O'Shea, 1999; Eils et al., 2000). The sites where nuclear proteins are maximally concentrated may or may not be the sites where they exert their function in pre-mRNA processing. Only a fraction of splicing factors would be expected to be engaged in splicing at any given time, and therefore the immunological staining patterns must be due to bulk splicing factors that are in the process of recycling, in reserve or otherwise engaged.

By analogy, the bulk of nuclear p66/ACF sedimented more slowly than 27S, leaving open the possibility that the localization of p66/ACF with heterochromatin represents free p66/ACF or complexes of p66/ACF smaller than 27S. In this case, the role of heterochromatin-associated p66/ACF is unclear, although it is generally held in the field that auxiliary proteins have additional roles in the cell (Schock et al., 1996; Smith et al., 1997). We cannot rule out the alternative explanation that editosomes are associated with heterochromatin but that nuclear p66/ACF dissociated during biochemical fractionation, leading to its recovery in fractions ≤27S.

The potential biological significance of two populations of editing factors

This is the first report in which the 60S complex has been characterized as a predominantly cytoplasmic assembly. The data suggested that editing factors were maintained in an inactive state in the cytoplasm through their physical associations as 60S complexes and are functional in the nucleus only as 27S editosomes. Although the bulk of p66/ACF was cytoplasmic in resting liver, the amount of functionally active nuclear editing factors could be increased in response to agents that stimulate editing activity. This raises important new questions of what regulates the proportion of cytoplasmic and nuclear p66/ACF (and APOBEC-1) and whether the cytoplasmic and nuclear pools of editing factors communicate through the exchange of proteins.

It is important to keep in mind that 60S complexes are not active in editing apoB mRNA in vivo (Yang et al., 2000) but can be activated under the dissociating conditions of the in vitro editing assay (Harris et al., 1993). Under these conditions it was shown that isolated 60S complexes dissociated to form functional 27S editosomes. In theory therefore, the 60S complexes in the cytoplasm could dissociate under appropriate metabolic stimuli to liberate editing factors or 27S editosomes for nuclear import.

Given that editing does not occur in the cytoplasm, the 60S complexes must maintain APBOEC-1 and p66/ACF in an inactive state through inhibitors or post-translational

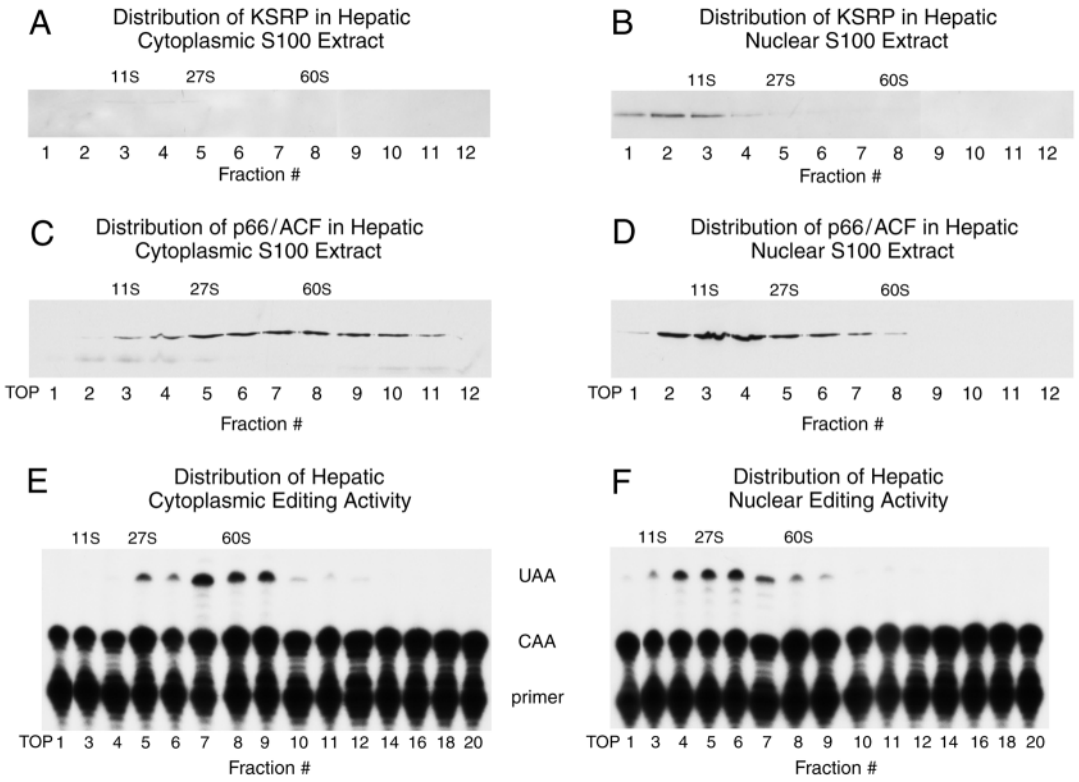


Fig. 10. Cytoplasmic and nuclear complexes containing editing factors. Cytoplasmic and nuclear S100 extracts were sedimented through 10%-50% glycerol gradients, fractionated and assayed for p66/ACF and KSRP by western blotting or for co-sedimenting APOBEC-1 by assaying in vitro editing activity. Glycerol gradients were loaded with 60 and 20 mg of cytoplasmic and nuclear S100 extracts, respectively. Gradient fractions from cytoplasmic (A,C,E) or nuclear (B,D,F) S100 extracts were analyzed by western blotting. Fractions are numbered from the top of each gradient and the gradient positions corresponding to 11S, 27S and 60S complexes are indicated. An equal aliquot of each gradient fractions was resolved by SDS PAGE and blotted. A and B are blots reacted with antibodies specific for KSRP; blots C and D were reacted with antibodies against p66/ACF. Poisoned primer extension and gel analysis of in vitro editing activity in gradient fractions from cytoplasmic (E) and nuclear (F) S100 extracts correspond to those immunoblotted in panels A/C and B/D, respectively. The primer extension products from unedited (CAA) and edited (UAA) RNA are indicated. The percent editing in each fraction was determined as described in Fig. 5 and was 2%, 2%, 16%, 14% and 14% for fractions 5-9, respectively, in E, and 15%, 15%, 17%, 4% and 0.5% in fractions 4-8 in F, respectively.

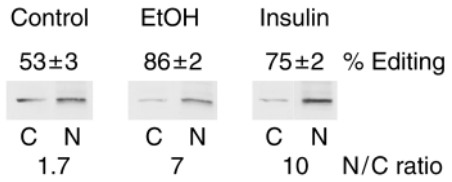


Fig. 11. Editing activity and the proportion of total cellular p66/ACF in the nucleus are enhanced following ethanol and insulin treatment. RNA was extracted from cultures of rat primary hepatocytes treated with ethanol or insulin for 6 hours. Editing activity was quantified by the poisoned primer extension assay, whereas cultures treated in parallel were subfractionated into cytoplasm proteins and nuclear proteins and assayed for p66/ACF by western blotting. Editing activity is shown as the average of three experiments ± s.e.m. The relevant regions of western blots for cytoplasmic proteins (C) and nuclear proteins (N) are shown and were prepared as described in Fig. 5. The nuclear to cytoplasmic (N/C) ratio was determined as described in Fig. 5 and did not vary more than 10% within treatment groups in the three replicate experiments.

modifications, or both. Candidates for this form of regulation are proteins such as αI3 serum proteinase inhibitor (Schock et al., 1996) that sequester editing factors or GRY-RBP (Blanc et al., 2001) and hnRNP proteins (Greeve et al., 1998) that inhibit APOBEC-1 catalytic activity.

The association of p66/ACF with the outer surface of the endoplasmic reticulum (ER), but not significantly with other cytoplasmic organelles, is of interest in terms of known protein trafficking mechanisms. There is ample evidence for the intracellular redistribution of proteins active in the nucleus from their sites of sequestration in the cytoplasm (Li et al., 1994; Rupp et al., 1994; Li et al., 1997; DeBose-Boyd et al., 1999; Görlich and Kutay, 1999; Haze et al., 1999; Kauffman and O'Shea, 1999). The endoplasmic reticulum frequently serves as the site where proteins are sequestered. Their release may be signaled by chaperones, covalent modification and/or proteolysis.

In this regard, apoB mRNA may serve as a means of tethering p66/ACF to the outer surface of the ER. Polysomes containing apoB mRNAs had an aberrantly slow sedimentation in glycerol gradients that was dependent on the presence of the mooring sequence (Chen et al., 1993). It has been proposed that the aberrant sedimentation may result from the persistent association of editing factors with the mooring sequence. In fact, there are 15 mooring sequences proximal to C6666 (Smith, 1993), and four of these have been shown to have associated editosomes and hence bound p66/ACF (Backus and Smith, 1991; Navaratnam et al., 1991; Backus et al., 1994). Consequently, there may be multiple p66/ACF bound to each apoB mRNA in the cytoplasm. Furthermore, arrested apoB mRNA translation complexes are docked on the ER of nonstimulated liver where they await appropriate metabolic stimulation to resume synthesis of apoB and subsequent formation of lipoprotein particles (Mitchell et al., 1998; Pariyarath et al., 2001).

Taken together, the immunolocalization of p66/ACF as clusters on the exterior surface of the ER could be a consequence of multiple p66/ACFs associated with apoB mRNA-ribosome translation complexes. This possibility is intriguing because release of p66/ACF for nuclear translocation might be accomplished through the resumption of translation, covalent modification of p66/ACF and/or release following ribonuclease activity. The mechanism(s) whereby p66/ACF accumulates in the nucleus following ethanol and insulin stimulation of editing is the subject of our ongoing research.

Trafficking, or the movement of proteins between intracellular compartments in response to specific signals, may explain the increase in nuclear p66/ACF in response to ethanol and insulin. The mechanism regulating p66/ACF nuclear import might involve an increase in apoB mRNA synthesis that occurs coincident with the stimulation of editing activity by insulin and ethanol (Thorngate et al., 1994; Funahashi et al., 1995; Lau et al., 1995). The expression of more mooring sequences in the nucleus could serve as a mechanism for recruiting p66/ACF to the nucleus by providing additional intranuclear binding sites. The abundance of unedited apoB mRNA in the nucleus, by itself, is not likely to recruit p66/ACF.

Shuttling of some of the proteins involved in RNA processing has been described as the movement of nuclear proteins to the cytoplasm in association with exported RNAs and subsequent return to the nucleus via their own NLS (nuclear localization signal) or in association with chaperones (Kauffman and O'Shea, 1999; Kim et al., 2001). In other words, the process may be continually ongoing and dynamic. Some splicing factors shuttle by a carrier-mediated pathway independent of RNA trafficking (Gama-Carvalho et al., 2001). Given that p66/ACF's association with apoB mRNA is mooring-sequence dependent but is not influenced by whether the mRNA is edited or not (Harris et al., 1993), it seems unlikely that the accumulation of nuclear p66/ACF in response to ethanol or insulin stimulation could be due to an increased export to the cytoplasm of edited apoB mRNA. It is possible that once established, an equilibrium of cytoplasmic and nuclear p66/ACF (and APOBEC-1) could be maintained through some form of shuttling.

Recent studies have suggested that APOBEC-1 requires a chaperone for its nuclear localization (Yang et al., 2001). The studies presented here suggested that APOBEC-1 is associated with p66/ACF throughout the cell and therefore it may import to the nucleus as an APOBEC-1-p66/ACF complex. A bipartite nuclear localization signal is predicted in p66/ACF (and ASP). However, the role of p66/ACF or other auxiliary proteins as a nuclear chaperone for APOBEC-1 awaits further proof.

In summary, the molecular cloning of p66/ACF and the production of peptide-specific antibodies reactive with this protein on western blots and tissues has enabled several important advancements in the apoB mRNA editing field. Our data have simplified the concepts in the field by showing that ACF is the well-studied 66 kDa, mooring-sequence-selective, RNA-binding protein that interacts early with apoB mRNA during editosome assembly. We also showed that functional complexes of p66/ACF and APOBEC-1 in the nucleus have a higher order complexity of 27S, thereby validating the biological relevance of in vitro assembled 27S editosomes. Native APOBEC-1 and p66/ACF are shown to also be in the cytoplasm of hepatocytes, where their physical interactions as 60S complexes are proposed to lead to their inactivation. Importantly, apoB mRNA editing and the relative abundance of p66/ACF in the nucleus and cytoplasm hepatocytes were metabolically regulated. These findings suggest that the

intracellular distribution of editing factors and their assembly as 27S or 60S complexes may be an important strategy in the metabolic regulation of apoB mRNA editing.

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