The Essential Vertebrate ABCE1 Protein Interacts with Eukaryotic Initiation Factors*

The ABCE1 gene is a member of the ATP-binding cassette (ABC) multigene family and is composed of two nucleotide binding domains and an N-terminal Fe-S binding domain. The ABCE1 gene encodes a protein originally identified for its inhibition of ribonuclease L, a nuclease induced by interferon in mammalian cells. The protein is also required for the assembly of the HIV and SIV gag polypeptides. However, ABCE1 is one of the most highly conserved proteins and is found in one or two copies in all characterized eukaryotes and archaea. Yeast ABCE1/RLI1 is essential to cell division and interacts with translation initiation factors in the assembly of the pre-initiation complex. We show here that the human ABCE1 protein is essential for in vitro and in vivo translation of mRNA and that it binds to eIF2α and eIF5. Inhibition of the Xenopus ABCE1 arrests growth at the gastrula stage of development, consistent with a block in translation. The human ABCE1 gene contains 16 introns, and the extremely high degree of amino acid identity allows the evolution of its introns to be examined throughout eukaryotes. The demonstration that ABCE1 plays a role in vertebrate translation initiation extends the known functions of this highly conserved protein. Translation is a highly regulated process important to development and pathologies such as cancer, making ABCE1 a potential target for therapeutics. The evolutionary analysis supports a model in which an ancestral eukaryote had large number of introns and that many of these introns were lost in non-vertebrate lineages.

The induction of ribonuclease L (RNase L) represents an important viral defense mechanism of mammalian cells against RNA viruses (1, 2). RNase L is present in the cell in an inactive form and can be activated by interferon. Interferon causes the activation of oligoadenylate synthases producing 2'-5' oligoadenylate. Bisbal et al. (3) described the isolation of a 68-kDa protein that binds to and inhibits RNase L and cloning of the gene. Although originally termed RNase L inhibitor (RLI), the gene is part of the ABC multigene family and its gene symbol is ABCE1. ABCE1 is induced during infection of cells with HIV-1 and an antiviral con-struct directed against ABCE1 resulted in a reduction of viral load (4). In cell-free extracts HIV-1 gag protein can assemble into viral capsids (5). This process is ATP-dependent and was shown to require a 68-kDa protein (HP68) identified as ABCE1/RLI. This same protein also functions in the assembly of HIV-2 and SIVmac (6).

Most of the ABC family genes encode large transport proteins that contain 6–17 transmembrane domains (7). ABCE1 is one of four human ABC genes that contain only nucleotide binding domains and are therefore not likely to be transporters. Of the 48 human ABC proteins, ABCE1 is the most conserved with a single copy of the gene in every characterized eukaryote, except for Arabidopsis, which has two ABCE1-like genes. In addition, there is an ABC-related gene in all characterized archaea but not in prokaryotes, demonstrating that this is one of the most conserved genes and is likely the ancestral ABC gene. Because the interferon system is not found outside of mammals, ABCE1 must have another function. Dong et al. (7) documented in Saccharomyces cerevisiae that ABCE1 (known as RLI1) is essential, and we found that yeast spores deficient in RLI1 do not undergo a single cell division. RLI1 binds to the eukaryotic initiation factors eIF2 and eIF5 and forms part of the pre-initiation complex required for the translation of mRNA in yeast. In addition, depletion of RLI1 causes a loss of polysome formation in yeast (7). The N terminus of the ABCE1 protein contains a Fe-S binding domain, and the protein binds Fe-S clusters (8). RLI1 was also found to be required for the processing of ribosomal subunits (9). Yeast RLI1 was able to rescue the lethal phenotype in the RLI1 gene deletion in yeast spores, whereas the human ortholog was not able to do so. Therefore, the question remained as to whether the mammalian ABCE1 plays a role in protein synthesis. We present in this report that human anti-ABCE1 antibody specifically inhibits mRNA translation, and ABCE1 protein binds to eIF2α and eIF5 initiation factors. Suppressing ABCE1 expression in a human cell line changes the polysome profile, with a decrease in large polysomes and an increase in 80S ribosomal subunits. We also show that ABCE1 is essential in embryo development of Xenopus laevis, another vertebrate model.

MATERIALS AND METHODS

Cell Culture—HeLa and HEK 293 cell lines (American Type Culture Collection, Manassas, VA) were grown in normal DMEM (glucose and glutamine) supplemented with 10% (v/v) fetal calf serum and 25 μg gentamicin/ml at 37 °C in a humidified atmosphere of 95% air and 5% CO2. All culture medium and reagents were obtained from Invitrogen. Cell counting was performed in a hemocytometer and Beckman Coulter cell counter, according to the vendor’s instructions.

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§ The abbreviations used are: RNase L, ribonuclease L; ABC, ATP-binding cassette; siRNA, small inhibitory RNA; RLI, RNase L inhibitor; HIV-1, human immunodeficiency virus, type 1; DMEM, Dulbecco’s modified Eagle’s medium; BisTris, 2-(bis(2-hydroxyethyl)-amino)-2-(2-hydroxyethyl)propane-1,3-diol; MO, morpholino oligonucleotide.
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To determine whether the human ABCE1 protein plays a role in the translation of mammalian mRNAs, we incubated rabbit reticulocyte lysates with an antibody to human ABCE1. To confirm that the human ABCE1 antibody reacts with the rabbit ABCE1 protein, we performed an immunoprecipitation with rabbit reticulocyte lysate. The human ABCE1 antibody precipitates a single protein of the expected size (Fig. S1). Luciferase mRNA was added to the lysate as a template along with different quantities of ABCE1 antibody. Fig. 1A shows that, at 200 ng of antibody, luciferase activity was inhibited by 90% compared with the no-antibody control. Rabbit IgG was not effective in inhibiting translation of luciferase mRNA even at 10-fold higher concentrations than the ABCE1 antibody. A human eIF5 antibody also suppressed luciferase mRNA translation initiation.

To demonstrate that the effect of ABCE1 on luciferase translation is specific for mRNA, we tested the effect of ABCE1 antibody on the translation of poly(U). Translation of poly(U) is accomplished independent of all initiation factors. The addition of ABCE1 antibody, at levels that severely inhibit translation of luciferase mRNA, had no significant effect on the translation of poly(U) (Fig. 1B). Therefore, the ABCE1 protein is an essential factor in vertebrate translation initiation.
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FIGURE 1. A, translation of LUC mRNA is inhibited by anti-ABCE1 antibody (Ab). LUC mRNA (4 ng, 1 μl) was used in 12.5-μl reaction mixtures containing 8.75 μl of rabbit reticulocyte lysate, 0.125 μl of amino acid (-Met), 0.125 μl of amino acid (-Leu), 0.25 μl of RNasin (40 units/μl), 1.25 μl of H2O, and 1 μl for antibody or controls. The amount of LUC mRNA was identified to be in a linear range with translated LUC activity (not shown). Antibodies or controls were pre-incubated with reaction mixtures at room temperature for 15 min, and then 1 μl of LUC mRNA was added and incubated for 90 min at 30 °C. Translation mixtures (1 μl) and a 40-μl LUC assay system were incubated for 20 min at room temperature and measured for luminescent signal. The assay of anti-ABCE1 antibody at 4 ng was performed in duplicate, all other assays were in triplicate. In the assay of eIF5 antibody, 1 μl of antibody at 10 times dilution was used. B, [3H]Phenylalanine incorporation into poly(phenyalanine) using poly(U) as a template. The reaction mixture contained 8 μl of rabbit reticulocyte lysate (Promega), 0.25 μl of RNasin (40 units/μl), 0.0625 μl of MgCl2 (1 M), 2.7 μl of [3H]phenylalanine (μCi/μl), 0.5 μl of PolyU (20 μg/μl), 1 μl antibody or 1 μl phosphate-buffered saline in a 12.5 μl total volume incubated at 30 °C, 90 min. The assay was performed in triplicate. The incubation mixtures were diluted with 10.5 μl of water and subjected to trichloroacetic acid precipitation on Whatman No. 3MM papers (24). Radioactivity incorporated was measured by liquid scintillation spectroscopy. C, immunoprecipitation analysis with anti-ABCE1, -eIF2α, and -eIF5 antibodies. HeLa cells in 75-cm2 flasks were lysed with 1 ml of protein extraction buffer, and 0.5-ml supernatants were reacted with 1 μl of anti-ABCE1 antibody, 1 μl of anti-eIF2α antibody, 1 μl of anti-eIF5 antibody, and 1 μl of pre-immune rabbit serum, respectively, for immunoprecipitation. The immunoprecipitation products were subjected to Western blot analysis. Left panel, anti-eIF5 antibody (2000× dilution) reacted with proteins immunoprecipitated by anti-eIF2α, -eIF5, and -ABCE1 antibodies, respectively. Lane 1 contains 100 ng of purified eIF5 protein. Right panel, anti-ABCE1 antibody (1000× dilution) reacted with proteins immunoprecipitated by anti-eIF5, -eIF2α, and -ABCE1 antibodies, and -pre-immunoprotein, respectively. All of second antibody reactions were diluted 10,000 times. The blots were detected by the ECL kit.

co-immunoprecipitated with eIF5 and to a lesser extent with eIF2α (Fig. 1C). Using an eIF2α antibody for detection we were also able to determine that eIF2α protein interacts with both eIF5 and ABCE1 (data not shown). Therefore, as in S. cerevisiae, ABCE1 associates with both eIF5 and eIF2.

To further study the role of ABCE1 on mammalian cell growth, we employed siRNA to reduce the expression of the ABCE1 gene in a human tumor cell line (HEK 293). The sequences and position in the mRNA of six pairs of siRNA are shown in Table 1. We titrated the capability of these siRNAs to suppress ABCE1 gene expression and to avoid off-target effects, we used low concentrations of siRNA. Transfection with four of siRNAs (at 5–10 nM) for 49 h suppressed ABCE1 mRNA expression more than 70%. At 72 h after transfection we carried out protein analysis of ABCE1. Immunoblotting showed that siRNAs 203097 and 203098, at 10 nM, substantially reduced ABCE1 protein expression (Fig. S2).

To evaluate the effect of suppression of ABCE1 expression on protein synthesis, the polysome profile was analyzed in cells treated with 10 nM siRNA 203097. In these cells, the large polyosomes were significantly reduced, with a commensurate increase in free 80 S ribosomes (Fig. 2C). This shift from polyosomes to monosomes is consistent with a severe impairment of translation initiation. The inhibition of the rate of translation initiation would be predicted to have profound effects on cell viability. Fig. 4A shows that 7 days of transfection with siRNA 203097 the proliferation of the cells was dramatically decreased. From day 3 to 7, the cell number of siRNA transfected cells was only increased 18%. However, the cell number of control samples was increased 320 to 350% and nearly reached confluence in a 24-well plate. Morphologically the transfected cells were clearly less dense, and there were many rounded, dying cells (data not shown). Therefore, suppression of ABCE1 expression inhibits cell proliferation in a human tumor cell line. Total incorporation of 35S into methionine and cysteine was also measured and found to be reduced by over 90% (Fig. 2B).

We have established a conserved function for ABCE1 in the protein translation process and provided evidence that it is critical for the proliferation of human cells in culture. Thus, we would expect an essential role for this protein in vertebrate development. To test this concept, two antisense MOs were designed and injected into X. laevis embryos. These two MOs were injected at various concentrations into two-cell stage embryos and allowed to develop in culture. One MO spanned the ATG start codon sequence, thus inhibiting mRNA translation. The other MO traversed the splice acceptor site and effectively suppressed proper
processing of ABCE1 mRNA (data not shown). Both MOs consistently arrested embryonic development at the gastrulation stage (Fig. 3). As expected, a control MO had no effect on development. These data demonstrate that ABCE1 is essential for vertebrate development as it is for yeast and \textit{Caenorhabditis elegans}. While this work was in progress a recessive lethal mutation in the \textit{Danio rerio} (zebrafish) genome was identified (17). In addition, mutations in CG1703 the \textit{Drosophila} ortholog have also been described that are lethal (18). Therefore ABCE1 is essential to all examined eukaryotes.

The ABCE1 gene is highly conserved, not only in eukaryotes, but a copy of the gene is found in all archaea. To determine the extent of amino acid conservation of the ABCE1 gene, sequences were extracted from databases for 21 eukaryotic genes and 24 archaeal genes. The sequences were aligned and used to produce an unrooted neighbor joining phylogenetic tree. All of the eukaryotic genes cluster as expected and provide a relationship of species consistent with other analyses. The only exceptions are the nematode species (\textit{C. elegans}, \textit{Caenorhabditis briggsae}) whose ABCE1 sequence clusters with the single celled eukaryotes (Fig. S3). This indicates that the ABCE1 gene in these species has evolved more rapidly. Within archaea, all of the ABCE1 genes from the Crenarchaea and Euryarchaea cluster together, and the one sequence from a Nanoarchaea sequence appears to be the most divergent.

Because of the very high level of conservation of the ABCE1 gene, and the fact that it is present as a single copy, it is one of the few genes that can be used to unambiguously study the evolution of intervening sequences. As expected none of the archae ABCE1 genes have introns, whereas all of the eukaryotic sequences have at least one intron, except for \textit{S. cerevisiae}.

**TABLE 1**

Sequence and location of ABCE1 siRNAs

Reference sequence ABCE1 (NM_002940).

| siRNA   | Sequence                     | Position bp | Exon   |
|---------|------------------------------|-------------|--------|
| 203097  | UCAUCAAACCUCAGAUUGU         | 617/635     | Exon 6 |
|         | ACAUAUGAGGUUCAAAUGA         | 718/736     | Exons 7 and 8 |
| 203099  | AUGUCACGACGCUUGAAUUAA       | 736/754     | Exon 8 |
| 2030999 | ACCGACCUAAAAGACAGA          | 186/204     | Exon 2 |
| 12213   | GGAAUGCAAAAAAGAUGUGG        | 424/442     | Exon 5 |
| 12306   | GGUGAAAGUUGGGAGUAG          | 2837/2855   | Exon 18 |
| 12394   | GGCAAACAUAAUGCAAGG          | 2837/2855   | Exon 18 |

**FIGURE 2.** A, inhibition of HEK 293 cell proliferation by siRNA. Cells (1 × 10^6/ml, 0.5 ml) were transfected with siRNA 203097 (at 10 nM final concentration) and control #1 siRNA with 3 μl of HiPerfect transfection reagent in 24-well plates, using the reverse transfection procedure. The assay was performed in triplicate. After 48-h transfection, the medium was changed to normal growth medium for an additional 24 h. On the 3rd day the cells were detached using 250 μl of trypsin and neutralized by 750 μl of normal medium (10% FCS). The cells were counted, and 7.4 × 10^4 cells were subcultured in 0.5 ml of medium in 24-well plates. All of the assays were done in triplicate. The cell number was counted at the indicated time. B, 2 × 10^4 treated and control cells in 100 μl of growth medium were seeded in different wells of 96-wells plate. After 48-h incubation at 37 °C/CO₂ in normal growth medium, the cells were washed with DMEM lacking methionine/cysteine with 2% (v/v) dialyzed FCS once. Then 80 μl of washing medium containing 12 μCi of [35S]methionine/cysteine was added to each well. After labeling, the cells in each well were lysed in 80 μl of protein lysis buffer. The 20-μl lysate was used for the assay of counts/min measurement. All of the assays were performed in triplicate. Blank control was no cells. The counts/min of all samples represents counts/min of sample minus counts/min of blank control. C, polysome profile. Top, Cytoplasmic extracts (12.1 A_{260} optical density) from control cells were sedimented in a sucrose gradient. Middle, control #1 siRNA-treated cells. Bottom, 10 nM siRNA 203097-treated cells.
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FIGURE 3. Antisense ABCE1 morpholino oligonucleotides antisense to the initiation (ATG) codon inhibits X. laevis embryonic development. A, antisense ABCE1 morpholino oligonucleotide injection. Fertilized eggs were injected with 80, 40, and 20 ng of oligonucleotide and 80 ng of control morpholino oligonucleotide, respectively. Each group contained 11 embryos. After 12-h incubation at 23 °C, all 11 embryos injected with 80 or 40 ng of oligonucleotide injection were unable to form a yolk plug, and 9 of 11 embryos injected with 20 ng of oligonucleotide were unable to form a yolk plug. All 11 embryos given control morpholino injection and un.injected controls formed clear yolk plugs. B, two representative control embryos from development stage 39 are also shown.

DISCUSSION

The ABCE1 gene is the most conserved member of the ABC gene family and is one of the most conserved genes in vertebrate and archaeal genomes (19). This fact alone suggests that the gene plays an essential role in biology that is common between archaean and eukaryotes. In addition, null mutations in the gene are homozygous lethal in every organism that has been examined. In this paper we found that ABCE1 is essential in Xenopus and that suppression of translation or splicing with morpholino oligonucleotides results in the cessation of growth of the embryo during gastrulation, a period when the germ layers of the embryo are formed and the body plan of the mature organism is established. ABCE1 mRNA was detectable by reverse transcription-PCR in oocytes, and growth cessation presumably occurs at the point at which most of the maternal protein has degraded.

The ABCE1 protein was originally identified due to an interaction with and inhibition of RNase L, a nuclease induced by interferon (3). However, RNase L is not found outside of vertebrates, indicating that ABCE1 has alternate functions. The identification of the role of the RLI/ABCE1 protein in ribosome biogenesis and in assembly of the pre-initiation complex of the ribosome in S. cerevisiae provides a function that is both essential and universal to eukaryotes (7). Therefore this is likely to be the original role of ABCE1 and the protein has adapted interaction with RNase L as a secondary function. However, we cannot rule out additional roles for ABCE1 in the cell.

The mammalian ribosome is substantially different from the yeast ribosome. For example the initiation complex component eIF3 has 14 subunits in mammals and only 6 in yeast. Therefore the importance of ABCE1 in mammalian protein initiation required experimental evidence. The important role of ABCE1 in protein synthesis is extended by the data presented here showing that the protein is essential in in vitro and in vivo translation of mammalian proteins. Antisera to ABCE1 block in vitro translation of mRNA in rabbit reticulocyte lysates but not of poly(U) molecules that can be translated independent of initiation factors. As in yeast, ABCE1 interacts with the eukaryotic initiation factors eIF5 and eIF2 components of the pre-initiation complex. Inhibition of ABCE1 in human cells results in dramatic inhibition of growth, reduction in the amount of large polysomes, and incorporation of labeled amino acids into newly synthesized protein. This is consistent with the results in yeast and supports a critical role for ABCE1 in the initiation of translation.

A single copy of the ABCE1 gene is found in all eukaryotes, except for Arabidopsis, which has two. This provides an excellent situation for the analysis of the phylogenetic relationships between species. From our analysis of a large number of eukaryotic and archaean species ABCE1 reproduces the known relationships between species in both kingdoms. The only exception is the nematodes C. elegans and C. briggsae in which the gene seems to be evolving more rapidly. The ABCE1 gene in C. elegans (Y39E4B.1) is essential and may have acquired other interacting partners that have driven its evolution (20). The conservation of ABCE1 also allows for an analysis of the evolution of intervening sequences in eukaryotes. All vertebrate ABCE1 genes contain 16 introns, except for some fish that have an additional 17th intron. The introns in the 5′-half of the gene are largely conserved in the plant (Arabidopsis) ABCE1 gene, but the introns in the 3′-half of the gene are in different locations in Arabidopsis as compared with vertebrates. All of the insect and nematode ABCE1 genes contain vastly reduced numbers of introns (2–6), and the introns they do contain are rarely shared, except for closely related species. For example the honey bee, mosquito, and fruit fly share...
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only one intron. However, all of the 11 introns found in the insect and nematode genes are in identical positions (and frames) as one of the vertebrate introns. This strongly supports the model derived from other genes that an ancestral eukaryote contained a large number of introns, many of which have been lost in certain lineages (21).

The inhibition of ABCE1 could have therapeutic applications. Because the protein is essential to most or all eukaryotes, specific inhibitors could be used in the treatment of pathogen. For example, inhibitors specific to plasmodia, fungi, and/or protozoan parasites could be used to inhibit such organisms as they infect human or other animals. We have shown here that ABCE1 inhibitors efficiently suppress the growth of human tumor cells. It is known that tumor cells have a high capacity for protein translation, and proteins involved in translation such as 6 kinase, mTOR, and 4E-BP1 are molecular targets for cancer therapy (22, 23). It is possible that cancer cells are more sensitive to inhibition of protein translation through ABCE1 than are normal cells. Last, ABCE1 is required for the assembly of HIV-1 and other lentiviruses (5). Drugs that interfere with the HIV/ABCE1 interaction could be used as antiretroviral agents.

The ABCE1 protein is unusual in containing a Fe-S cluster binding site. It has long been known that Fe-S clusters are assembled in the mitochondria, and this process is essential to the cell. ABCE1 clearly represents one essential Fe-S containing protein. Whether this is the only essential protein in this class remains to be determined.

In summary we have demonstrated that the ABCE1 protein plays a role in the initiation of translation of proteins, similar to its role in yeast. We also show that this highly conserved gene is essential to Xenopus development and the growth of human cells, just as it is to yeast, C. elegans, Drosophila, and zebrafish. The ABCE1 gene is one of the few genes conserved between archaean and eukaryotes and can therefore be used for phylogenetic analysis of species and the evolution of introns.

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FIGURE 4. Intron analysis of the ABCE1 gene. The introns were located in the genetic sequence of the ABCE1 ortholog from all available eukaryotic species. The introns are numbered consecutively across the top, and the number of introns in each species is shown following the species name. For each intron the amino acid residue involved in the splice is shown with phase 0 introns in yellow, phase 1 in green, and phase 2 in blue.