Reconstitution of mRNA editing in yeast using a Gal4-apoB-Gal80 fusion transcript as the selectable marker

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Summary

We describe a fusion transcript of Gal4 linked to its specific inhibitor protein Gal80 by 276 nucleotides of apolipoprotein (apo) B sequence a selectable marker for mRNA editing. Editing of apo B mRNA is catalyzed by an editing enzyme-complex that introduces a stop codon by deamination of C to U. The catalytic subunit APOBEC-1 is a cytidine deaminase and requires a second essential component recently cloned and termed APOBEC-1 complementing factor (ACF) or APOBEC-1 stimulating protein (ASP). The aim of this study was to demonstrate that APOBEC-1 plus ACF/ASP comprises all that is required for editing of apo B mRNA in vivo. Expression of APOBEC-1 and Gal4 fused to its inhibitor Gal80 by an intervening unedited apo B sequence (Gal4-apoB_C-Gal80) did not result in the Gal4 dependent expression of HIS3 and β-galactosidase in the yeast strain CG1945. Co-expression of APOBEC-1 and ACF/ASP induced editing of the apo B site in up to 13% of the Gal4-apoB_C-Gal80 transcripts and enabled selection of yeast cells for robust expression of HIS3 and β-galactosidase. Additional expression of the alternative splicing regulatory protein KSRP increased the editing of the apo B site by APOBEC-1 and ACF/ASP to 21%. Thus, APOBEC-1 and ACF/ASP represent the core apo B mRNA editing enzyme in vivo. This study demonstrates for the first time the successful use of a selectable marker for mRNA editing. The gal4-gal80 system is analogous to the two-hybrid assay and may have broader application for the study of other mRNA processing reactions.
Introduction

mRNA editing is a genetic regulation that alters gene expression by posttranscriptional nucleotide changes within the coding regions of transcripts. mRNA editing of nuclear encoded genes consists of site-specific deamination reactions that convert A to I and C to U. In most cases, these base changes result in important functional alterations of the edited gene products (1,2). The A to I editing described for glutamate receptors, serotonin 5-HT\textsubscript{2C} receptors and the hepatitis delta virus RNA is mediated by a family of adenosine deaminases known as ADARs (adenosine deaminases acting on RNAs) (3,4). These enzymes function as single peptides and contain both RNA-binding and deaminase activity (3). C to U editing has been described in plant mitochondria, in Physarum polycephalum and also in mammals (2,4).

The best characterized example of C to U editing occurs in the mRNA of apolipoprotein (apo) B that creates a premature stop codon and leads to the synthesis of the truncated apo B-48 (5,6). The apo B mRNA is extensively edited in the human small intestine, but remains unedited in the liver that secretes apo B-100 containing very-low-density lipoproteins (VLDL) as precursors for the atherogenic low-density lipoproteins (LDL) (7,8). Some animals such as dog, horse, rat and mouse do edit the apo B mRNA also in the liver and consequently have very low plasma LDL levels (7). Thus, editing of the apo B mRNA is a decisive genetic regulation for the formation of atherogenic lipoproteins (7).

The editing of apo B occurs coincident with splicing and polyadenylation of the mRNA and is mediated by the apo B mRNA editing enzyme-complex (9-11). An 11-nucleotide “mooring” motif downstream of the editing site from nucleotide position 6671-6681 is absolutely required for the editing reaction (12). The catalytic component APOBEC-1 is a cytidine deaminase that deaminates C to create U (13-16). APOBEC-1 requires other components for its editing activity (13-16). Activation-induced deaminase (AID), a close homologue of APOBEC-1 cloned from antigen stimulated germinal center B-lymphocytes, induces class switch recombination, somatic hypermutation and immunoglobulin gene conversion in developing B-lymphocytes (17-21). AID is supposed to be an mRNA editing enzyme, although this has not been proven so far (22).
In 2000 the cloning of the second essential component of the apo B mRNA editing enzyme-complex was reported by Driscoll and co-workers and simultaneously by our group (23,24). This protein termed APOBEC-1 complementing factor (ACF) by Driscoll et al. and APOBEC-1 stimulating protein (ASP) by us represents a novel type of RNA-binding protein with three non-identical binding domains for single-stranded RNA at the aminoterminus and a putative binding domain for double-stranded RNA at the carboxyterminus (23). Purified recombinant ACF/ASP and purified recombinant APOBEC-1 reconstitute very strong apo B mRNA editing in vitro (23,24). Two variants of ASP/ACF differing by an eight amino acids insertion that have identical activity and various other splice variants have been described that are produced by alternative splicing of the ACF/ASP gene (24-26). While the ADARs that mediate A to I editing contain the RNA-binding motifs and the catalytic domains in the same polypeptide, in C to U editing these modular elements for an editing enzyme appear to be separated into APOBEC-1 and ASP/ACF (24).

In our protein purification that resulted in the cloning of ASP, the KH-type splicing regulatory protein (KSRP) co-purified and demonstrated stronger RNA-binding to apo B mRNA than ASP, suggesting that KSRP might also contribute to editing of apo B mRNA (24,27). Three other proteins have been recently isolated by two-hybrid selection in yeast using APOBEC-1 as the bait that also have been implicated in the editing of apo B mRNA (28-30). Earlier biochemical studies already suggested that the apo B mRNA editing enzyme-complex may consist in high molecular weight complexes (24,31). Therefore, although it was demonstrated by several studies that APOBEC-1 and ASP/ACF exert strong apo B mRNA editing activity in vitro, it is unknown whether APOBEC-1 and ASP/ACF are all that is required for editing of apo B mRNA in vivo.

To demonstrate that APOBEC-1 and ASP/ACF represent the core of the apo B mRNA editing enzyme-complex that is fully competent to edit apo B mRNA in vivo, we set out to establish an editing system in yeast. This eukaryotic organism was chosen since it is genetically most distant to mammals in which this type of mRNA editing has evolved (2,4). A fusion transcript of the yeast transcription factor Gal4 linked to its specific inhibitor protein Gal80 by an intervening apo B fragment was used as a selectable marker. We demonstrate that the apo B mRNA editing enzyme-
complex is reconstituted in yeast by expression of APOBEC-1 and ASP/ACF. mRNA editing introduces a stop codon in the Gal4-Gal80 fusion transcript and allows to select for expression of active Gal4 protein. This is the first example that mRNA editing is reconstituted in yeast, and the selection system presented may have broader application for the study of other mRNA editing or processing reactions.

**Experimental Procedures**

**Plasmids:** *pAS-GAL4-ApoB-GAL80:* Apo B cDNAs with a C or a T at nucleotide position 6666, respectively, were amplified by PCR using oligonucleotides L/NcoI (GCCATGGATATACAAATTGCA TTAGATGATG, nt 6591-6615 plus NcoI site) and L/BspH1rev (GTCATG AAT CCAAGA TGCAGTACT ACT TCC, nt 6867-6842 plus BspH1 site), cloned into pGEM-T Easy (Promega) and sequenced. The apo B cDNA was excised from the vector by NcoI/BspH1 restriction enzyme digest and cloned into the unique NcoI-site of construct pSB32-GAL4(1-841)-GAL80 kindly provided by Dr. Stephen A. Johnston, University of Texas, Southwestern Medical Center, Dallas, Texas, USA. The resulting construct GAL4-ApoB-GAL80 was amplified by PCR using oligonucleotides Gal4-s (CAAGCTTATGAAGCTACTGTCTTCTATCGAAC) and Gal80-rev (GGATCCAGCAATCTCGATCGAATTAATGTCGC), cloned into pGEM T Easy and entirely sequenced to confirm the nucleotide sequence. pAS-GAL4-ApoBc-GAL80 (pAS-Gal4C) and pAS-GAL4-ApoBu-GAL80 (pAS-Gal4U), respectively, were generated by tri-fragment ligation using (i) the HindIII-BamHI fragment of pGEMT-GAL4-ApoB-GAL80 containing the GAL4-ApoB-GAL80 construct, (ii) the HindIII-SacI fragment, and (iii) the SacI-BamHI fragment of pAS2 (Clontech). The correct composition of the final constructs was confirmed by DNA sequencing.

*pLS317-Apobec-1:* Rat Apobec-1 cDNA was excised from pSVL-Apobec-1 (32) with EcoRI and BamHI and cloned into the EcoRI-BamHI site of pPGK2. The PGK-promoter along with the full
length Apobec-1 cDNA was excised with XhoI and SalI and cloned into the SalI site of pLS317 (obtained from ATCC) to generate pLS317-Apobec-1.

*pACT-APOBEC-1*: pACT–APOBEC-1 was constructed by tri-fragment ligation using (i) an HindIII-SacI fragment containing the open reading frame of rat APOBEC-1, (ii) the HindIII-ScaI and (iii) the ScaI-SacI fragment of pACT2 (Clontech). In the resulting pACT-APOBEC-1, the nuclear localization signal and the activation domain of GAL4 are deleted, and APOBEC-1 is under the control of the ADH promoter and is followed by the ADH termination signal (pACT∆AD-APOBEC-1).

*pACT-KSRP*: pACT-KSRP was constructed by cloning an EcoRI-XhoI fragment containing the full length KSRP cDNA into the EcoRI-XhoI sites of pACT-APOBEC-1 thereby replacing APOBEC-1 with KSRP.

*pACT-ASP*: pGEM-T Easy containing the full length cDNA of ASP with the insertion of 8 amino acids (24) was digested with SacII and was treated with T4 DNA polymerase. After phenol/chloroform extraction and ethanol precipitation the ASP cDNA was excised by restriction enzyme digestion with SalI. pACT-APOBEC-1 was digested with EcoRI and treated with T4 DNA polymerase. After phenol/chloroform extraction and ethanol precipitation it was further digested with XhoI. The full length ASP cDNA was inserted into pACTII∆AD by ligating the SalI-XhoI sites and the blunted EcoRI and SacII sites, respectively.

*pBridge-GAL4-ApoB-GAL80*: The Gal4-ApoB-Gal80 cassettes of pAS-Gal4-ApoB<sub>C</sub>-Gal80 and pAS-Gal4-ApoB<sub>U</sub>-Gal80, respectively, were excised by restriction enzyme digestion with XhoI and BamHI and inserted into the XhoI and BamHI sites of pBridge to generate pBridge-Gal4-ApoB<sub>C</sub>-Gal80 and pBridge-Gal4-ApoB<sub>U</sub>-Gal80, respectively.

*pBridge-Gal4-ApoB<sub>C</sub>-Gal80-ASP*: The full length cDNA of ASP was excised from pGEM-T Easy with NotI and inserted into the unique NotI site of the multiple cloning site II of pBridge-Gal4-ApoB<sub>C</sub>-Gal80 to generate pBridge-GAL4-ApoB<sub>C</sub>-GAL80-ASP. The resulting construct was sequenced to confirm the correct orientation of ASP.
pBridge-Gal4-ApoBC-Gal80-KSRP: The full length cDNA of KSRP was excised from pGEM-T Easy by restriction enzyme digest with NotI and inserted into the unique NotI site of the multiple cloning site II of pBridge-Gal4-ApoBC-Gal80 to generate pBridge-Gal4-ApoBC-Gal80-KSRP.

**Transformation and growth conditions of yeast CG1945:** The yeast expression plasmids were transformed into the yeast strain CG-1945 (Clontech) by standard methods as described previously (33). The genotype of CG1945 is MATa, ura3-52, his 3-200, lys2-801, ase2-101, trp1-901, leu2-3, 112, Gal4-542, Gal80-538, cyh2, Lys2::Gal1UAS-Gal1TATA-HIS3; URA3::GAL4 17mers(X3)-CyC1TATA-lacZ. The LYS2 gene is non-functional. Yeasts were grown on synthetic drop-out media as described (33). Synthetic media lacking histidine were supplemented with 5 mM 3-amino-1,2,4-triazole (3AT) as a competitive inhibitor of the His3 protein in order to inhibit low levels of His3 protein expressed in a leaky manner and thus to suppress background growth. Filter assays for β-galactosidase were performed using standard methods as described (33).

**Analysis of apo B mRNA editing in yeast:** Total RNA was prepared from yeast by acid phenol extraction. The yeast were grown to late log phase on synthetic drop-out media either with or without histidine in the presence of 5 mM 3-AT. The apo B cassette of the Gal4-ApoB-Gal80 transcript was amplified by RT-PCR with oligonucleotides GAL4-1 sense (CTTTCAACAACCAATTGCCTCCTCTAAC) and apoB2 (CACGGATATGATAGTGCTCATCAAGAC) and analyzed for editing by primer extension assay (7,32). Primer extension products were quantitated as described (7,32).
Results

Gal4-apoB-Gal80 as a selectable marker for mRNA editing in yeast: The yeast transcription factor Gal4 is inhibited by complex formation with its specific inhibitor Gal80. A fusion protein consisting of Gal4 and Gal80 is inactive and does not promote Gal4 dependent transcription. To establish a functional assay system for apo B mRNA editing in vivo, we reasoned that this Gal4-Gal80 fusion transcript might be a useful selectable marker for mRNA editing in yeast. Therefore, we inserted 92 amino acids of the apo B sequence that encompass the editing site at C_{6666} in frame between Gal4(1-841) and Gal80 to generate Gal4-apoB_{C}-Gal80 (Gal4-C). As a positive control we constructed Gal4-apoB_{U}-Gal80 (Gal4-U) that contains the edited version of the apo B sequence with the premature stop codon. Gal4-C should not be able to promote GAL4 dependent transcription because of complex formation with its specific inhibitor GAL80, while the edited version Gal4-U that contains only a tail of 25 amino acids of apo B sequence should be active similar to wild-type GAL4 (Fig. 1).

Gal4-C and Gal4-U were expressed in yeast CG1945 cells using the expression plasmid pAS in the absence or presence of APOBEC-1 (Fig. 2 A). The yeast strain CG1945 is commonly used for two-hybrid selection. It contains the lacZ- and the HIS3-gene under the transcriptional control of Gal4. Expression of active Gal4 can be monitored by growth in the absence of histidine and by detection of β-galactosidase activity. After transformation of pAS2-Gal4-U and the empty vector pLS317 the yeast cells grew well on media lacking tryptophane, lysine and histidine, and exhibited strong β-galactosidase activity (Fig. 2 B). In contrast, yeast CG1945 cells transformed with pAS2-Gal4-C and the control vector pLS317 did not grow on media without histidine, and failed to express β-galactosidase (Fig. 2 B). Even after co-transformation of pAS2-Gal4-C and pLS317-APOBEC-1 the yeast cells did not grow without histidine and did not express β-galactosidase activity (Fig. 2 B). To analyze the apo B editing site the Gal4-C and Gal4-U transcripts were amplified by RT-PCR and analyzed for editing by primer extension assay (Fig. 2 C). The Gal4-U transcripts from yeast cells transformed with pAS2-Gal4-U contained -as expected- only U at apo B position 6666, and the Gal4-C transcript from yeast transformed with
pAS-Gal4-C and pLS317 contained -as expected- only C at this position (Fig. 2 C). The Gal4-C transcripts from yeast transformed with pAS-Gal4-C and pLS317-APOBEC-1 did not differ and also did not contain significant amounts of U residues at the editing site (Fig. 2 C). These results confirmed the predictions that Gal4 is active in yeast when expressed from the "edited" Gal4-U since the stop codon prevents the translation of Gal80, but is inactive when expressed from Gal4-C because of complex formation with Gal80. Moreover, expression of only APOBEC-1 is not sufficient to induce mRNA editing at the apo B site of Gal4-apoBc-Gal80.

mRNA editing in yeast by co-expression of APOBEC-1 and the APOBEC-1 stimulating protein ASP: It was further investigated whether a co-expression of APOBEC-1 with ASP (ACF) or with KSRP would lead to a functional editing enzyme-complex in yeast that could induce a stop codon at the editing site of Gal4-C and thereby enable the cells to express active Gal4. For these experiments Gal4-C and Gal4-U were inserted into the yeast expression plasmid pBridge to generate pB-Gal4-C or pB-Gal4-U, respectively. pBridge allows the expression of a second gene from the Met25 promoter that is induced in the absence of methionine (Fig. 3 A). Yeast CG1945 cells were transformed with pLS317-APOBEC-1 and either pB-Gal4-U, pB-Gal4-C, pB-Gal4-C/ASP or pB-Gal4-C/KSRP (Fig. 3 A) and were grown on media lacking tryptophane, lysine and methionine. The apo B cassettes of the Gal4-C or Gal4-U mRNAs from the transformed yeast cells were amplified by RT-PCR and analyzed for editing by primer extension assay (Fig. 3 B). The positive control Gal4-U contained only U, while Gal4-C contained only C at the editing position (Fig. 3 B). Approximately 12% of the C residues in the apo B sequence of Gal4-C from yeast cells transformed with pB-Gal4-C/ASP were found to be edited and were sequenced as Us (Fig. 3 B). No Us at this position of the apo B sequence could be detected in yeast cells that were transformed with pB-Gal4-C/KSRP (Fig. 3 B).

These yeast cells that had been grown on media lacking tryptophane, lysine and methionine to select for the presence of the two plasmids and to induce expression from the meth 25 promoter were streaked onto nylon membranes and grown on media lacking also histidine. As
anticipated from the editing assay, only the yeast cells transformed with either the positive control Gal4-U or with Gal4-C/ASP grew on these media and exhibited activity of β-galactosidase (Fig. 3 C). Therefore, in yeast the apo B site in Gal4-C is edited in the presence of APOBEC-1 and ASP. This mRNA editing leads to the expression of active GAL4 and thus enables the cells to grow in the absence of histidine and to express β-galactosidase.

mRNA editing in yeast by co-expression of APOBEC-1, ASP and the KH-type splicing regulatory protein KSRP: Next it was studied whether additional expression of KSRP would enhance the editing efficiency achieved by APOBEC-1 and ASP alone. For this experiment, we constructed a third yeast expression plasmid, pACT, that allows the expression of ASP or KSRP separate from the expression of Gal4-C (Fig. 4 A). Yeast CG1945 cells were triple-transformed with pLS317-APOBEC-1, pAS-Gal4-C or pAS-Gal4-U, and pACT-ASP, pACT-KSRP or empty control pACT, and were grown on media lacking tryptophane, lysine and leucine. Editing of the apo B site in Gal-4C was analyzed by primer extension assay of RT-PCR amplified Gal4-C mRNA (Fig. 4 B). As before, the positive control pAS-Gal4-U contained only U at the editing position, while no U residue could be detected at this position in yeast transformed with pLS317-APOBEC-1, pAS-Gal4-C and pACT-KSRP or the empty vector control pACT (Fig. 4 B). In yeast cells transformed with pAS-Gal4-C, pLS317-APOBEC-1 and pACT-ASP, however, approximately 8% of the C residues at the apo B editing site demonstrated successful editing and were sequenced as Us in the primer extension assay (Fig. 4 B). This result proved that expression of APOBEC-1 from pLS317 and of ASP from pACT is sufficient to induce competent editing activity in yeast CG1945 cells.

In the next experiment the three presumptive components of the apo B mRNA editing enzyme-complex were expressed in yeast CG1945 cells using the three yeast expression plasmids pB-Gal4-C, pLS317-APOBEC-1 and pACT-ASP or KSRP (Fig. 5 A). The apo B cassettes were amplified by RT-PCR from yeast cells grown on media lacking tryptophane, lysine, leucine and...
methionine, and were analyzed for editing by primer extension assay (Fig. 5 B). While the positive control pBGal4-U contained only U at the editing position, no U could be detected at this position in yeast cells transformed with pLS317-APOBEC-1, with pB-Gal4-C and the empty control pACT or pACT-KSRP (Fig. 5 B). In yeast cells transformed with pLS317- APOBEC-1, pB-Gal4-C and pACT-ASP, approximately 13% of the C residues of the apo B site were edited and analyzed as Us in the primer extension assay (Fig. 5 B). This amount of editing was further increased to 21% in yeast cells that were transformed with pB-Gal4/ASP and pACT-KSRP (Fig. 5 B). The triple-transformed yeast were re-streaked onto synthetic media lacking -in addition to tryptophane, lysine, leucine and methionine- also histidine. Only the positive control with pB-Gal4-U and the yeast cells transformed with pB-Gal4-C plus pACT-ASP or with pB-Gal4-C/ASP plus pACT-KSRP grew in the absence of histidine and demonstrated robust ß-galactosidase activity (Fig. 5 C).

Discussion

The results of this investigation allow two important conclusions. First, it is demonstrated for the first time that coordinate expression of the catalytic subunit APOBEC-1 and the APOBEC-1 stimulating protein ASP/ACF is sufficient to create apo B mRNA editing in vivo. These two proteins therefore represent the minimal core components of the apo B mRNA editing enzyme-complex in vivo. These experiments were performed in yeast because this is the eukaryotic organism most distant to mammalians in which this type of mRNA editing has evolved to modulate lipoprotein metabolism. The efficiency of apo B mRNA editing in yeast mediated by APOBEC-1 and ASP (ACF) is limited and can be increased by the expression of the alternative splicing factor KSRP. Second, a tripartite fusion of the yeast transcription factor Gal4 with its
specific inhibitor Gal80 and an short intervening apo B sequence is demonstrated as a potent selectable marker for the functional study of mRNA editing in vivo. This approach using the Gal4-Gal80 fusion transcript should be more widely applicable and may be useful to study also other forms of mRNA editing or RNA processing. The system is based on the two-hybrid assay and requires only little modifications of the Gal4-Gal80 transcript depending on the special type of application.

The molecular identification of ASP/ACF by Driscoll and co-workers and simultaneously by our group immediately led to the question as to whether APOBEC-1 and ASP/ACF are sufficient for apo B mRNA editing in vivo (23,24). APOBEC-1 together with ASP/ACF exert strong apo B mRNA editing activity in vitro, but this does not necessarily imply that these two protein are all that is required for editing in vivo. This investigation was set out to address this question by reconstitution of apo B mRNA editing in yeast. We assumed that this would be the most direct approach to this issue. Even the generation of an ASP/ACF deficient mouse model in which presumably editing would be abolished could not rule out that other proteins besides APOBEC-1 and ASP/ACF are required for editing in vivo. Moreover, in our protein purification that led to the molecular cloning of ASP the alternative splicing factor KSRP co-purified with ASP (24). Although no effect of KSRP on apo B mRNA editing could be demonstrated in vitro, we were inclined to assume that this co-purification might indicate that these two protein do interact functionally in vivo. The main objective of our study was to demonstrate reconstitution of apo B mRNA editing in vivo that was not only detectable by analysis of the transcript per se, but also exerted a physiological effect.

A short apo B fragment containing the editing site was inserted in frame between a Gal4-Gal80 fusion protein (kindly provided by Stephen A. Johnston, University of Texas, Dallas,
USA) to create a selectable marker for apo B mRNA editing in yeast. Gal4 is inhibited by Gal80, when both proteins are expressed on the same polypeptide. The nuclear localization signal and the activation domain of Gal4 were deleted from the pACT expression plasmid to allow a coordinate expression of ASP. Gal4-C was a tight selectable marker that did not cause background growth of the yeast cells without editing. Even when APOBEC-1 was co-expressed, no editing of the apo B site was detectable and Gal4 was inactive. This result is in contradiction to another study that demonstrated APOBEC-1 mediated mRNA editing in yeast without any other additional exogenous component (34). The most likely explanation for this discrepancy is the level of APOBEC-1 expression. We chose the PGK promoter in order to avoid aberrant hyperediting by APOBEC-1 that has been demonstrated in cell culture in vitro and in transgenic animals in vivo (35,36). High level expression of APOBEC-1 by the galactose inducible GAL1 promoter and an exogenous SV40 nuclear localization signal at the aminoterminus apparently increases the nuclear abundance of APOBEC-1 and leads to editing of apo B transcripts even in the absence of ASP/ACF (34). This phenomenon was excluded in our experiments by moderate constitutive expression of APOBEC-1 mediated by the PGK promoter. In addition, the absence of editing in Gal4-C without APOBEC-1 and ASP/ACF does not favour the endogenous yeast gene CDD1 as an editing enzyme as has been previously proposed (37).

Apo B mRNA editing activity was reconstituted in yeast by the expression of APOBEC-1 and ASP/ACF and induced the expression of active Gal4 due to the introduction of a stop translation codon between Gal4 and Gal80. This was shown with two different expression plasmids. These results provide proof that APOBEC-1 and ASP/ACF represent the core of the apo B mRNA editing enzyme-complex that is missing in yeast. The extent of editing at the apo B site in Gal4-C that was achieved in yeast was limited to a maximum of 21%. This falls short from editing of apo B mRNA in the intestine where in every message C6666 is edited to U (5-7). Several reasons may explain this limitation of editing in yeast. First, the Gal4-C transcripts contain only 276 nucleotides of apo B sequence and may not be the ideal substrate for APOBEC-1 mediated mRNA editing. Second, it may well be that the optimum activity of the apo B mRNA editing enzyme-
complex in vivo requires additional components besides APOBEC-1 and ASP that are missing in yeast.

The co-expression of KSRP increased editing from a level of 13% achieved by APOBEC-1 and ASP alone to approximately 21%. This indicates that KSRP can contribute to editing of apo B mRNA in vivo. Its precise function in the editing process, however, remains to be studied, but this was not the aim of the present investigation. Recently, three other proteins, GRY-RBP, CUGBP2 and ABBP-2, have been identified by two-hybrid selection in yeast using APOBEC-1 as the bait, and have been suggested to be involved in the editing of apo B mRNA (28-30). GRY-RBP is an RNA-binding protein with approximately 50% homology to ASP/ACF which itself cannot support APOBEC-1 to edit apo B mRNA in vitro, but can inhibit the in vitro editing reaction (28). CUGBP2 is an ubiquitously expressed RNA-binding protein that can bind to apo B mRNA and to ACF/ASP, and has also the potential to inhibit in vitro editing (29). ABBP-2, a novel human Class II DnaJ homologue and member of the human Hsp40 family, was demonstrated not only to interact with APOBEC-1, but also to be required for editing of apo B mRNA in APOBEC-1 expressing BNLCL.2 cells (30). These results taken together suggest that editing of every Gal4-C transcript may require more components in addition to APOBEC-1 and ASP/ACF that comprise only the essential core of the editing enzyme-complex. The Gal4-C reporter system will be an ideal tool to analyze the effects of GRY-RPB, CUGBP2 or ABBP-2 on the editing efficiency of APOBEC-1 and ASP/ACF and may provide further proof for the necessity of any of these in the editing reaction.

This study is the first to demonstrate that transcriptional regulation by Gal40-Gal80 can be used to reconstitute and select for mRNA editing in yeast. Our system is based on the two-hybrid selection assay and uses the commercially available yeast strain CG1945 that is widely used for these studies. The nuclear localization signal and the activation domain of GAL4 were deleted from the commercially available yeast expression plasmid pACT, and the Gal4 binding domain in pAS and pBridge was replaced by the Gal4-apoB-Gal80 fusion construct. The Gal4-apoB-Gal80 selection marker does not produce background growth, and the synthesis of Gal4 is entirely
dependent on mRNA editing of the apo B site. We did not observe breakthrough of the selection and demonstrated that the Gal4-apoB-Gal80 transcripts were properly processed and not cleaved prematurely as an obvious alternative to activate Gal4 (R. Kirsten, I. Diehl and J. Greeve, unpublished observations). This system may therefore be used to further study the apo B mRNA editing-enzyme complex in yeast by analyzing mutants of APOBEC-1 and ASP/ACF. Moreover, by modifying the Gal4-Gal80 reporter this system may be adapted to investigate other mRNA processing reactions. Obvious applications include the analysis of mRNA cleavage terminating the transcript behind Gal4 and of alternative splicing that would remove Gal80. Our work therefore extends the use of the Gal4 transcription factor to the analysis of mRNA editing and processing and may be helpful to elucidate such processes.

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Figure legends

Figure 1: Illustration of the structure and function of the gal4-apoB-gal80 fusion transcripts. Gal4-apoB<sub>C</sub>-Gal80 (Gal4-C) containing the unedited apo B sequence leads to a fusion protein in which gal4 is inhibited by complex formation with Gal80. In contrast, Gal4-apoB<sub>U</sub>-Gal80 (Gal4-U) gives rise to active Gal4 protein due to a premature stop translation codon in the edited apo B sequence.

Figure 2: Transformation of pAS-Gal4-C or pAS-Gal4-U and pLS317-APOBEC-1 into yeast CG1945 cells. (A) Illustration of the plasmids pAS and pLS317. (B) Transformed CG1945 yeast cells were streaked onto synthetic media lacking either tryptophane and lysine or tryptophane, lysine and histidine in the presence of 5 mM 3-AT, and were grown for 24 h at 30°C (left panel). Nitrocellulose filter lift were taken, grown for another 12 h on the same synthetic drop-out media and analyzed by β-galactosidase filter assay (right panel). (C) Gal4 transcripts were amplified by RT-PCR from total RNA of transformed yeast CG1945 cells grown on synthetic drop-out media lacking tryptophane and lysine and analyzed for editing of the apo B sequence by primer extension assay. The extension products for edited (C) and unedited (U) apo B sequences are indicated.

Figure 3: Transformation of pLS317-APOBEC-1 and pBridge-Gal4-C/ASP into yeast CG1945 cells. (A) Illustration of the plasmids pBridge and pLS318. (B) Gal4 transcripts were amplified by RT-PCR from total RNA of transformed yeast CG1945 cells grown on media lacking tryptophane, lysine and methionine and analyzed for editing of the apo B sequence by primer extension assay. The extension products for edited (C) and unedited (U) apo B sequences are indicated. (C) Transformed yeast CG1945 cells grown on synthetic media without tryptophane, lysine and methionine were re-streaked onto synthetic media lacking tryptophane, lysine, methionine and histidine in the presence of 5 mM 3-AT. After growth for 24 h at 30°C, nitrocellulose filter lifts were taken, further grown for another 12 h and analyzed by β-galactosidase filter assays.
Figure 4: Triple transformation of pLS317-APOBEC-1, pAS-Gal4-C and pACT-ASP in yeast CG1945 cells. (A) Illustration of the plasmids pAS, pLS317 and pACT. (B) Gal4 transcripts were amplified by RT-PCR from total RNA of transformed yeast CG1945 cells grown on media lacking tryptophane, lysine and leucine and analyzed for editing of the apo B sequence by primer extension assay. The extension products for edited (C) and unedited (U) apo B sequences are indicated.

Figure 5: Triple transformation of pLS317-APOBEC-1, pAS-Gal4-C and pACT-ASP in yeast CG1945 cells. (A) Illustration of the plasmids pBridge-Gal4-C/ASP, pLS317 and pACT-ASP or pACT-KSRP. (B) Gal4 transcripts were amplified by RT-PCR from total RNA of transformed yeast CG1945 cells grown on media lacking tryptophane, lysine, leucine and methionine and analyzed for editing of the apo B sequence by primer extension assay. The extension products for edited (C) and unedited (U) apo B sequences are indicated. (C) Transformed yeast CG1945 cells grown on synthetic media without tryptophane, lysine, leucine and methionine were re-streaked onto synthetic media lacking tryptophane, lysine, leucine, methionine and histidine in the presence of 5 mM 3-AT. After growth for 24 h at 30°C, nitrocellulose filter lifts were taken, further grown for another 12 h and analyzed by β-galactosidase filter assays.
Fig. 1

mRNA Gal4 Apo B CAA Gal80

Gal4 / Gal80 inactive

mRNA Gal4 Apo B UAA Gal80

Gal4 active

Gal4-C

Gal4-U
Gal4-C or U + Apobec-1

CG 1945

A

pAS-Gal4-C + pLS317
pAS-Gal4-C
pAS-Gal4-U
pAS-Gal4-C + pLS317-Apobec-1

growth on -T/-LYS
growth on -T/-LYS/-H

β-Gal assay
β-Gal assay

B

pAS-Gal4-U + pLS317

pAS-Gal4-C + pLS317

C

100 <1 <1 % editing

pAS pLS317

pAS-Gal4-C + pLS317

pAS-Gal4-ApoB-Gal80

Apobec-1
Fig. 3

**Legend:**

**A**

Gal4-C or U/ASP or KSRP

Apobec-1

CG 1945

**B**

+ pLS317pBridge

+ pBridge

+ pBridge

+ pBridge

ß-Gal filter assay

yeast grown on -Tryp, -Lys, -Met, -His

**C**

6-Gal filter assay

yeast grown on -Tryp, -Lys, -Met, -His

% editing
Fig. 4

A

Gal4-C or U + Apobec-1 + ASP or KSRP

CG 1945

B

+ pAS-Gal4-C+pACT + pAS-Gal4-C+pACT + pAS-Gal4-C+pACT+ASP + pAS-Gal4-C+pACT+KSRP

- U

- C

100 <1 8 <1 % editing
Fig. 5

**A**

- Gal4-C or U/ASP or KSRP
- pBridge
- Apobec-1
- pLS317
- ASP or KSRP

**B**

- β-Gal filter assay
- Yeast grown on -Tryp, -Lys, -Leu, -Met, -His

**C**

- % editing
- U/C

100 <1 <1 13 21
Reconstitution of mRNA editing in yeast using a Gal4-apoB-Gal80 fusion transcript as the selectable marker
Heinrich Lellek, Sybille Welker, Ines Diehl, Romy Kirsten and Jobst Greeve

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