TIA-1 OR TIAR IS REQUIRED FOR DT40 CELL VIABILITY

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TIA-1 and TIAR are a pair of related RNA-binding proteins which have been implicated in apoptosis. We show that chicken DT40 cells with both tia-1 alleles and one tiar allele disrupted (tia-1(-/-)tiar(-/+)) cells are viable. However, their growth and survival in medium containing low serum levels is significantly reduced compared to DT40 cells. The remaining intact tiar allele in tia-1(-/-)tiar(-/+)) cells can only be disrupted if TIA-1 expression is first restored to the cells by transfection of a TIA-1 expression vector. We conclude that DT40 cells require either TIA-1 or TIAR for viability. TIA-1 overexpression in tia-1(-/-)tiar(-/+)) cells leads to a radical drop in TIAR levels, by inducing efficient splicing of two tiar alternative exons carrying in frame stop codons. In wild-type DT40 cells, tiar transcripts including these exons can also be detected. These transcripts increase significantly in abundance in cycloheximide-treated cells, suggesting that splicing of the exons exposes mRNAs to nonsense-mediated mRNA decay. TIA-1 or TIAR depletion leads to a marked drop in splicing of the exons. The human tiar gene contains a corresponding pair of TIA-1-inducible alternative exons, and we show that there is very high sequence conservation between chickens and humans of the exon pair and parts of the flanking introns. The TIA-1/TIAR responsiveness of these alternative tiar exons is likely to be of physiological importance for controlling TIAR levels.
INTRODUCTION

TIA-1 (1,2) and the TIA-1 related protein TIAR (3) are a pair of similar, ubiquitous RNA-binding proteins with three RNA recognition motifs (RRMs). These proteins fill important functions in both the cytoplasm and the nucleus, as do a number of other multifunctional regulatory proteins (4). In the cytoplasm, TIA-1 and TIAR control translation of some specific mRNAs (5-9), and are active in the general translational arrest mechanism induced by stress (10-15). TIA-1 and TIAR shuttle between cytoplasm and nucleus, but are predominantly nuclear in most cells studied. In the nucleus, they activate splicing of exons with weak 5' splice sites followed by uridylate-rich stretches (16-18). TIA-1 and TIAR appear to bind to these stretches (they both bind preferentially to uridylate-rich sequences in vitro (19)), and assist binding of U1 snRNP to the adjacent 5' splice site. Possible target exons include an fgfr-2 alternative exon (16), a Drosophila msl-2 exon (20), a human fas exon (20), and some alternative human tia-1 and tiar exons containing premature stop codons (21). TIAR and possibly TIA-1 are also involved in replication of the RNA genomes of West Nile virus (22).

Overexpression of TIA-1 or TIAR induces apoptosis, whether accomplished by exposure of permeabilised cells to recombinant proteins (1,3), or by infecting cells with an engineered virus (23). During Fas-induced apoptosis, TIA-1 is phosphorylated (24), and TIAR is relocated to the cytoplasm (25). TIA-1 activates splicing of a fas pre-mRNA exon so as to favour production of a cell death receptor, at the expense of a soluble form of Fas (17). Based on these observations, TIA-1 and TIAR have been proposed to be apoptosis-promoting proteins (17,20). On the other hand, approximately 50% of tia-1(-/-) mice die between embryonic day 16.5 and 3 weeks of age, though surviving mice appear normal (7). Mice with disrupted tiar genes show high levels of embryonic lethality, from 90-100% depending on the strain analysed (7,26). Mice with both tia-1 and tiar genes disrupted die before embryonic day 7 (7). These results may be a sign that TIA-1 or TIAR are required for some
specific process (possibly involving apoptosis) required for proper development, which, if not carried out, leads to early embryonic lethality. The results could thus be in agreement with the proposed apoptosis-promoting activity for TIA-1 and TIAR. Alternatively, they may indicate that the proteins are in fact necessary for cell viability. It seemed important to us to distinguish between these two possibilities.

One way to do so is to disrupt the genes in a cell line. Homologous recombination is very efficient in the chicken B-cell line DT40 (27,28), and this line has been used extensively for gene disruption studies (29-31). Using a gene disruption approach, we show here that DT40 cells expressing only TIA-1 or only TIAR are viable. However, we were unable to obtain cells expressing neither of the two proteins, suggesting a requirement for at least one of the TIA-1/TIAR pair for cell viability. We also show that elements of a tiar gene auto-control mechanism are highly conserved between chickens and humans, and discuss whether TIA-1/TIAR should be regarded as apoptosis promoting factors or cell survival factors.
MATERIALS AND METHODS

Cloning of chicken tia-1 and tiar genes

Chicken TIA-1 and TIAR ESTs were identified by interrogating a database (32) with the human TIA-1 and TIAR cDNA sequences. The corresponding TIA-1 cDNA clone (DKFZp426G159) was obtained from the Resource Center of the German Human Genome Project, Max-Planck-Institute for Molecular Genetics, Berlin, and sequenced. It coded for a truncated TIA-1, containing a deletion of 5 base pairs (AGCAG) at an exon-exon junction, which we attributed to inappropriate use of the underlined AG to mark the end of the intron during splicing. This defect was corrected by site-directed mutagenesis using the QuickChange kit from Stratagene. The corrected cDNA coded for a full-length TIA-1 protein. The chicken TIAR EST sequence identified (dkfz426_14A13R1) appeared to start within the intron upstream of exon 6, but also contained exon 6-11 sequences. Missing 5' sequences were obtained by carrying out RT-PCR on DT40 RNA using a downstream exon 8 primer (5'-GTAGCCCAAGCAAT-3') derived from the EST sequence, and an upstream primer (5'-ATGGAAGACGACGGGCAGC-3') identical to the human TIAR cDNA sequences coding for the first amino acids of human TIAR. Two amplified fragments were obtained, subcloned, and sequenced. They differed by the presence or absence of 51 base pairs, reflecting the use of two alternative 3' splice sites for exon 3. Missing 3' sequences were obtained from a composite sequence (044907.3) which became available subsequently, and which was derived by combining several ESTs (data from the UMIST/Nottingham/Dundee universities chicken EST sequencing project, http://chick.umist.ac.uk/). Our final composite chicken tia-1 cDNA sequences code for proteins of 388 and 371 amino acids. Tia-1 and tiar probes were used to screen a chicken cosmid genomic library (library 125 from the Resource Center of the German Human Genome Project Max-Planck-Institute for Molecular Genetics, Berlin), using instructions supplied by the library provider. Cosmid DNA was prepared from positive colonies. Maps of the chicken tia-1 and tiar genes were made using cosmid DNA by determining the sizes of PCR products obtained using primers within adjacent exons for amplification across the intervening intron. Sequences determined here (chicken TIA-1 and
TIAR cDNA sequences, and the chicken tiar gene sequences between exons 5 and 6 (including exons 5A and 5B), and between exons 10 and 11 (including exon 11A) are available under GenBank Accession Numbers AY189903-189906. Human TIA-1 and TIAR cDNA sequences are available under GenBank Accession Numbers M77142 and M96954 respectively. The human tiar gene sequence is available as EnsEMBL gene ENSG00000151923 at http://www.ensembl.org.

**Targeting vectors**

Various drug-resistance cassettes under control of the chicken β-actin promoter and linked to either an SV40 or a thymidine kinase gene polyadenylation signal were kindly provided by Jean-Marie Buerstedde. Targeting vectors as shown in Figs. 2-4 were made by standard subcloning procedures (33). The tia-1 gene targeting vectors contain either a 2.3 kb neomycin-resistance cassette, or a 2.3 kb puromycin-resistance cassette, flanked by tia-1 gene sequences (4.1 kb upstream, and 4.9 kb downstream). Tiar gene targeting vector 1 contains a hygromycin B-resistance cassette, flanked by tiar gene sequences (4.0 kb upstream, and 4.3 kb downstream). Tiar gene targeting vector 2 contains a mycophenolic acid-resistance cassette (gpt sequence). The gpt sequence is preceded by a 3' splice site and polyadenylation sequence taken from pCI-neo (Promega). This composite block is flanked by tiar gene sequences (2 kb upstream, and 2 kb downstream).

**DT40 cell electroporation**

DT40 cells were kindly provided by Jean-Marie Buerstedde and were maintained at 37°C and 5% CO₂, in RPMI 1640 (Sigma) containing 10% fetal calf serum, 1% chicken serum (Sigma), 2 mM L-glutamine (Sigma) and 100 μM β-mercaptoethanol (Sigma). For each transfection, 10⁷ cells were washed and resuspended in 800 ml of phosphate-buffered saline containing 30 μg of linearized plasmid, before electroporation using a Gene Pulser apparatus (Bio-Rad) set to 550V and 25 μF. After electroporation, cells were added to 20 ml of fresh culture medium, and 100 μl aliquots transferred to each well of a 96 well plate. After 24 h, 100 μl of fresh
medium containing the appropriate antibiotic was added. After 7 to 10 days, resistant cells were transferred to wells of a 24 well plate, and subsequently further expanded. The following final concentrations of antibiotics were used: G418, 2 mg/ml; puromycin, 0.5 µg/ml; hygromycin B, 1 mg/ml; histidinol, 1 mg/ml; mycophenolic acid, 25 µg/ml, added together with xanthine to 250 µg/ml, and hypoxanthine to 15 µg/ml. All antibiotics were from Sigma, except for G418, which was from Invitrogen.

**Growth tests**

Different cell lines were first cultivated for 48 h in the absence of any antibiotic selection. They were then seeded at a density of 3x10^5 cells/ml in antibiotic-free medium containing 1% chicken serum and either 0%, 1% or 10% fetal calf serum. Aliquots were removed after different times, and cells counted. Viable and non-viable cells were distinguished by eosin staining.

**DNA and RNA analysis**

Isolation of DNA and RNA, and Southern blotting were carried out using standard techniques (33). RT-PCR was carried out as described elsewhere (34,35). Primers used were 5'-GGAAGACGACGGGCAGCC-3' from tiar exon 1 with 5'-GCAAAGGCTGACTTGATA -3' from tiar exon 5; 5'-CAATCACTTTCCACGTGTTCG-3' from tiar exon 5 with 5'-GTTAGCCCAGAAGCAAT-3' from tiar exon 8; 5'-GTACTCATGGGAGGCCAG-3' from tiar exon 7 with 5'-AGGCTGAGCACCAATCCACCCAT-3' from tiar exon 12. 24 to 28 cycles of amplification (1' at 94°C; 1' at 50°C; 2'30 at 72°C) were used so as to remain in the range of exponential amplification. PCR products were migrated on 2% agarose gels, transferred to nylon filters (Hybond N+, Amersham), and hybridized with a ^32P-labelled probe corresponding to tiar exons 1-12 from the tiar cDNA. Quantitative PCR was done using the Brilliant Quantitative PCR Core Reagent Kit from Stratagene with cDNA from different lines, using conditions specified by the manufacturer. Reactions were carried out and analysed using
an MX-4000 apparatus from Stratagene. In this technique an intercalating dye is used to quantify PCR products. For each sample, two amplifications are carried out in parallel, one to quantify tiar products, and one to quantify chicken actin products, the latter quantification permitting comparison of different samples. Primers used were in tiar exon 5 (5'-CCACGTGTTCGTTGGGGATTTAAG-3') and tiar exon 5A (5'-GCACAGTTCTTGCAGTTAGCC-3') for quantification of exon 5A-containing RNA. For quantification of tiar RNA without any alternative exon spliced between exons 5 and 6, a primer from tiar exon 5 (5'-CTTCCACGTGTTCGTTGGG-3') and a primer crossing the tiar exon 5/6 junction (5'-CCGTGCATCCGATATTTTACC-3') were used. Actin primers were 5'-TGGGCTTCATCACCAACGT-3' and 5'-GCCCCAGACATCAGGGTGT-3'. Each primer pair yielded a single PCR product. Where indicated, cells were incubated in cycloheximide (50 µg/ml) for 2 h before harvesting for RNA preparation.

Western blotting

Cells were lysed by repeated cycles of freezing and thawing. Protein content of extracts was determined using the BCA-200 Protein Assay Kit from Pierce with bovine serum albumin as a standard. Aliquots of protein (30 µg) were subjected to 10% SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose, and incubated with goat polyclonal antibodies against TIA-1 or TIAR (Santa Cruz Biotechnology, Inc.), or mouse antibodies against ASF/SF2 (gift from James Stévenin). Detection was carried out with a POD kit from Roche.
RESULTS

Chicken TIA-1 and TIAR

DT40 cells express a 40 kDa TIA-1 singlet comigrating with the faster moving band of the human TIA-1 doublet (data not shown). This doublet results from alternative splicing of tia-1 exon 5 (36), so this suggests that DT40 TIA-1 mRNA does not contain an exon equivalent to human exon 5. A TIA-1 cDNA identified in a chicken bursal EST database (32) codes for a 372 amino acid protein 95% identical to the human TIA-1 form lacking exon 5-encoded sequences (see Materials and Methods). DT40 cell TIAR was detected as a 40 kDa doublet comigrating with the human TIAR doublet (data not shown). Both doublets arise from use of two alternative 3′ splice sites for tiar exon 3 ((3) and our unpublished observations). The amino acid sequence of chicken TIAR was deduced from sequences of cDNA fragments we amplified using RT-PCR on DT40 RNA (see Materials and Methods). The longer chicken TIAR isoform of 388 amino acids is 96% identical to the corresponding isoform of human TIAR.

Amplified TIA-1 and TIAR cDNA fragments were used to screen a chicken cosmid library, and several tia-1 and tiar cosmid clones were isolated. These were used to establish a map of the corresponding genes (Fig. 1A; note that exons are numbered following the assumption that there is no chicken equivalent of the alternatively spliced human tia-1 exon 5). A schematic representation of chicken TIA-1 and TIAR is shown in Fig. 1B. These proteins are composed of three RNA recognition motifs (RRMs) and a glutamine-rich domain. In the human proteins, the second RRM is required for (and indeed responsible for) their specific binding to uridylate-rich sequences (19). Given the very high similarity observed between the chicken and human proteins, it seemed safe to assume that the second RRMs in the chicken proteins play the same roles. These RRMs are encoded by exons 5 and 6 (note that we assume that there is no chicken counterpart to the human alternative exon 5, so chicken tia-1
exons 5 and 6 correspond to human tia-1 exons 6 and 7). We decided to disrupt the tia-1 and tiar genes by insertion of sequences in exon 5, so as to disrupt the sequences coding for RRM2, as shown in Fig. 1B (see arrow 'insert').

tia-1 gene disruption in DT40 cells

One of the tia-1 cosmid clones was used for construction of the targeting vectors shown in Fig. 2A, in which either a neomycin-resistance gene or a puromycin-resistance gene have been inserted in tia-1 exon 5. DT40 cells were electroporated with the neomycin-resistance targeting vector. DNA was isolated from 19 neomycin-resistant clones, digested by EcoRV, and analysed by Southern blotting with a tia-1 probe covering exons 6-10. This probe detects a 5.5 kb fragment in DT40 cell DNA (intact allele in Fig. 2A and in Fig. 2B, lane 1), and should detect an additional 7.8 kb fragment in clones with one disrupted allele (Fig. 2A). This latter result was observed for four clones (see Fig. 2B, lanes 2 and 3 for two examples of such tia-1(+/-) clones). One of the tia-1(+/-) clones was subsequently electroporated with the puromycin-resistance targeting vector. Out of 29 puromycin-resistant clones analysed as above, four appeared to have both tia-1 alleles disrupted: the 5.5 kb fragment representing an intact allele had disappeared, and only 7.8 kb fragments representing disrupted alleles were detected (see Fig. 2B, lanes 4 and 5 for two examples). Western blotting with antibodies against TIA-1 showed that these tia-1(-/-) clones no longer produce TIA-1 (Fig. 2C, lanes 3 and 4; the very weak doublet observed at 40 kDa is due to slight cross-reactivity of the antibodies with TIAR).

tiar gene disruption in DT40 cells

We made a tiar targeting vector (Fig. 3A) in which exon 5 was disrupted by insertion of a hygromycin B-resistance gene. DT40 cells were electroporated with this vector. DNA from
19 hygromycin B-resistant clones was digested by EcoRV and analysed by Southern blotting with a tiar probe covering the genomic region between exons 3 and 5. In DT40 cell DNA, the two tiar alleles are detected on separate EcoRV fragments of 12 kb and 7.6 kb (intact alleles 1 and 2 in Fig. 3A and Fig. 3B, lane 1). Successful targeting of one of these alleles should lead to complete loss of one of these EcoRV fragments. This was observed for one of the 19 hygromycin B-resistant clones analysed (Fig. 3B, lane 2). This clone was termed a tiar(1+/2-) clone to indicate that allele 2 has been disrupted. Both tia-1 alleles were disrupted in this clone by successive use of the two tia-1 targeting vectors shown in Fig. 2A. The resulting clone was named clone 3-2 (Fig. 3B, lane 3), as it contains three inactivated alleles: two tia-1 alleles, and tiar allele 2.

When tiar targeting vector 1 was electroporated into tia-1(-/-) cells, a tiar allele (allele 1 this time) was inactivated in one of the 19 hygromycin B-resistant clones tested (Fig. 3B, lane 4). This clone was named clone 3-1, as it contains three inactivated alleles: two tia-1 alleles and tiar allele 1. Note that a different tiar allele is disrupted in clones 3-1 and 3-2. All of the clones discussed above contain at least one intact tiar allele, and continue to express TIAR as a doublet (Fig. 3C).

We attempted without success to disrupt the remaining intact tiar allele in clone 3-1 using a targeting vector similar to that shown in Fig. 3A, but containing a mycophenolic acid-resistance or a histidinol-resistance cassette in place of the hygromycin B-resistance cassette (a total of 26 histidinol-resistant clones and 39 mycophenolic acid-resistant clones were analysed). While this result is consistent with a requirement for TIA-1 or TIAR for cell viability, no conclusion can be drawn from it, as the targeting efficiency of the tiar vectors used is not very high (see previous two paragraphs).
A second *tiar* targeting vector

We tried to improve targeting efficiency using a vector with a mycophenolic acid-resistance cassette (gpt sequence) designed to target a different region of the *tiar* gene. Targeting vector 2, and the expected results of successful *tiar* gene disruption using it, are shown in Fig. 4A. In any allele disrupted by recombination with this vector, a 3' splice site followed by two polyadenylation signals (pA) will be found in the intron following exon 2. The disrupted allele can only yield a truncated mRNA containing exons 1 and 2, followed by part of intron 2, and so will be incapable of furnishing functional TIAR. Note that in clones 3-1 and 3-2, the vector can target either the intact *tiar* allele (to generate a gpt disrupted allele), or the allele already disrupted by insertion of the hygromycin B-resistance gene (to generate a hygro+gpt disrupted allele).

Targeting vector 2 was electroporated into clones 3-1 and 3-2, and DNA from mycophenolic acid-resistant clones was subjected to EcoRV digestion and analysis by Southern blotting with a probe corresponding to exon 2 and 2 kb of downstream intron sequences. For the clone 3-2 electroporation, we analysed 30 clones. The intact *tiar* allele 1 was disrupted in none of these clones. However, in 6 of them the hygro disrupted allele 2 had been targeted a second time, to yield cells with an intact allele 1 and a hygro+gpt disrupted allele 2 (3-2* cells, see Fig. 4B, lane 2 for an example). For the clone 3-1 electroporation, we analysed 55 clones. Once again, the intact *tiar* allele 2 was disrupted in none of these clones, but in 9 of them the already hygro disrupted allele 1 had been targeted a second time, to yield cells with an intact allele 2 and a hygro+gpt disrupted allele 1 (3-1* cells, see Fig. 4C, lane 2 for an example).
Both tiar alleles can be disrupted only in cells expressing TIA-1

With targeting vector 2, only clone 3-1 and 3-2 cells in which an already disrupted tiar allele has been targeted a second time were obtained. This strongly suggests that cells expressing neither TIA-1 nor TIAR are not viable. However, we could not rule out that the already disrupted allele was much more susceptible to targeting than the intact allele for some reason. We felt that it might be possible to disrupt the remaining intact tiar allele if TIA-1 expression was first restored. We prepared clone 3-1 cells re-expressing TIA-1 as a result of stable transfection with an epitope-tagged TIA-1 expression vector (clone 3-1+TIA-1 cells). These cells express levels of TIA-1 significantly higher than those expressed by DT40 cells (Fig. 2C, compare lanes 5 and 6 to lane 1). The cells were electroporated with targeting vector 2 (Fig. 4A), and mycophenolic acid-resistant clones were selected. In 4 of the 46 clones analysed, the already disrupted tiar allele 1 had been targeted a second time (data not shown). However, in 4 other clones the intact allele 2 had been targeted, to yield cells with no intact tia-1 allele and no intact tiar allele (clone 4+TIA-1 cells, see Fig. 4C, lane 3 for an example). These results demonstrate that the intact allele is as susceptible to targeting as the already disrupted allele. Clone 4+TIA-1 cells do not contain TIAR (Fig. 4D, lane 5; the very weak band at 40 kDa is due to cross-reaction of the antibodies used with the epitope-tagged TIA-1).

In summary, electroporation of targeting vector 2 into cells with both tia-1 alleles and one tiar allele disrupted led to isolation of 15 clones with the already disrupted allele targeted a second time, but no clones with the intact tiar allele targeted, despite the fact that intact and disrupted tiar alleles are equally susceptible to targeting. However, tia-1(-/-)tiar(-/-) cells expressing TIA-1 from an integrated expression vector can be obtained readily. This is a very strong indication that tia-1(-/-)tiar(-/-) cells are not viable, and shows in addition that TIA-1 can functionally replace TIAR for viability. As tia-1(-/-) cells are viable, TIAR can also replace TIA-1 for viability.
Reduced growth and survival of tia-1(-/-)tiar(-/+) cells in low serum

As tia-1(-/-)tiar(-/-) cells are not viable, we wondered if the viable tia-1(-/-)tiar(-/+)
cells (clones 3-1 and 3-2) had more stringent requirements for growth or survival than DT40
cells. We tested growth of various lines in medium containing 0%, 1% or 10% fetal calf serum
(and 1% chicken serum). The results of a typical series of experiments are shown in Fig. 5.
There is no significant difference in growth between DT40 cells, tia-1(-/-) cells, tiar (+/-) cells,
cloned 3-1 cells and clone 3-2 cells in medium containing 10% fetal calf serum (Fig. 5A and B).
In medium containing 1% or 0% fetal calf serum there is significant mortality of DT40 cells,
tia-1(-/-) cells and tiar(+/-) cells, but these cells continue to proliferate, and the net result is an
increase in the number of viable cells with time (Fig. 5A). However, for clones 3-1 and 3-2,
the number of viable cells remained almost stationary in medium containing 1% fetal calf
serum, and actually dropped rapidly in medium containing 0% fetal calf serum (Fig. 5B),
with a concomitant increase in the number of dead cells observed (data not shown). This
suggests that the tia-1(-/-)tiar(-/+). cells are unable to survive under these reduced serum
conditions. This defect was however eliminated (Fig. 5B) when tia-1(-/-)tiar(-/+). cells were
forced to re-express TIA-1 from an expression vector (we used the clone 3-1+TIA-1 cells
described above). Clone 4+TIA-1 cells behaved like DT40 cells under all the conditions tested
(data not shown).

TIA-1 overexpression prevents TIAR expression by inducing splicing of exons with stop
codons

While analysing the expression of TIAR in clone 3-1+TIA-1-derived cells, we noticed
that clone 3-1+TIA-1 does not express TIAR (Fig. 4D, compare lanes 2 and 3), despite the
fact that it contains one intact tiar allele. Clone 3-1+TIA-1 overexpresses TIA-1 as compared
to DT40 cells (Fig. 4D, compare lanes 1 and 3). We also analysed a clone 3-1+TIA-1
derivative (clone 3-1*+TIA-1, see above), in which the already targeted tiar allele 1 has been targeted a second time, but which still contains one intact tiar allele. In this derivative, TIA-1 levels had dropped to levels similar to those seen in DT40 cells, and TIAR expression was restored (Fig. 4D, lane 4). These results suggest that TIA-1 overexpression prevents TIAR expression.

We have shown previously that TIA-1 overexpression induces the splicing of alternative human tiar exons with premature stop codons (21), although we did not investigate the effect of this on TIAR levels. We wondered if this mechanism was conserved in chicken cells, and so studied TIAR mRNA expression patterns in clones 3-1 and 3-1+TIA-1 by an RT-PCR approach. Using primers in exons 1 and 5, little difference was observed between the two clones (Fig. 6A, compare lanes 1 and 2; two fragments were obtained as there are two alternative 3' splice sites for exon 3). However, we detected some RT-PCR products (products a, b and c) present only in the clone 3-1+TIA-1 sample when we used a primer pair in exons 5 and 8, or a primer pair in exons 7 and 12 (Fig. 6A, compare lanes 3 and 4, and lanes 5 and 6, respectively). These products were not detected in RNA from clone 3-1*+TIA-1 (data not shown).

Note that the results shown for lane 4 result from a 12-fold longer exposure of the film than that used for lane 3. This is a reflection of the fact that TIAR mRNA levels are much lower in clone 3-1+TIA-1 cells than in clone 3-1 cells (as discussed below, and see Fig. 7C for quantification of this difference), while RNAs with exons 5A and 5B included are unstable. The new products (marked a, b and c in Fig. 6A) were cloned and sequenced, and their sequences compared to the chicken TIAR cDNA sequence (data not shown), and to selected parts of the chicken tiar gene. Comparison of these sequences showed that product b is the result of splicing of two additional exons (5A and 5B) between exons 5 and 6. In product a, the intron between exons 5A and 5B is retained. Product c is the result of splicing of an
additional exon (10A) between exons 10 and 11. All these additional exons contain in-frame stop codons (Fig. 6B), and their splicing explains why clone 3-1+TIA-1 does not contain detectable levels of normal TIAR. The 5' splice sites of exons 5A and 5B are followed by a uridylate-rich sequence, as expected for TIA-1 activated sites (Fig. 6B). This is not the case for exon 10A, however.

**TIA-1/TIAR is required for exon 5A splicing**

RT-PCR analysis of RNA from DT40 cells with primers in tiar exons 5 and 8 shows that these cells contain very low levels of mRNAs with exons 5A and 5B included (Fig. 7A, lane 1). This could be because inclusion of these exons with their in-frame stop codons exposes resulting mRNAs to nonsense-mediated mRNA decay (37). To test this, we incubated DT40 cells in the presence of the protein synthesis inhibitor cycloheximide to block nonsense-mediated mRNA decay. This treatment lead to a significant increase in the levels of TIAR mRNAs containing exon 5A spliced between exons 5 and 6, or both exon 5A and exon 5B spliced between exons 5 and 6 (Fig. 7A, compare lanes 1 and 2). This was confirmed (Fig. 7B) by carrying out quantitative RT-PCR on RNA from DT40 cells incubated or not in cycloheximide, using primers in exons 5 and 5A as shown in Fig. 7B. Use of this primer pair will lead to amplification of the same DNA fragment from RNA with exon 5A spliced between exons 5 and 6, and from RNA with both exons 5A and 5B spliced between exons 5 and 6. The primer pair can thus be used to quantify total exon 5A splicing.

In contrast, cycloheximide treatment of DT40 cells did not induce any significant increase in normal TIAR mRNA levels in DT40 cells or clone 3-1 cells (Fig. 7C), as judged by quantitative RT-PCR using a primer in exon 5 together with a primer crossing the exon 5-exon 6 junction of normal TIAR mRNA (this primer pair should detect TIAR mRNA with exon 6 spliced to exon 5, but not mRNA with exons 5A or 5B spliced to exon 5). Very low levels of
normal TIAR mRNA were detected in clone 3-1+TIA-1 cells (Fig. 7C). This result is not surprising, as in these cells exons 5A or 5B are spliced very efficiently.

To test for the effects of TIA-1/TIAR depletion on exon 5A splicing, quantitative RT-PCR was carried out on RNA from various cell lines with disrupted genes (incubated or not in cycloheximide) using primers in exons 5 and 5A to detect exon 5A-containing mRNAs (Fig. 7B). Note that in Fig. 7B results shown represent the levels of exon 5A-containing mRNAs obtained from one tiar allele, i.e. we have corrected for the fact that some of the cell lines tested have one tiar allele, while others have two. As shown in Fig. 7B, TIA-1 depletion leads to a drop in exon 5A splicing (tia-1(-/-) cells). A more marked drop is observed in tiar(+/-) cells lacking one tiar allele. In cells lacking both tia-1 alleles and one tiar allele (clones 3-1 and 3-2), splicing of exon 5A falls to very low levels (see also Fig. 7A, compare lanes 3 and 4 for clone 3-1, and lanes 5 and 6 for clone 3-2). Exon 5A splicing is thus truly responsive to TIA-1/TIAR levels, decreasing when levels fall, and increasing when levels rise.

What is the magnitude of the effect that fluctuations of exon 5A splicing can have on TIAR protein levels? It should be noted that in DT40 cells, about 30% of TIAR mRNAs contain exon 5A (Fig. 7A, lane 2). So if no exon 5A splicing at all were to take place in DT40 cells for some reason, TIAR mRNA and protein levels would be expected to increase by a maximum of about 1.4-fold. A lower increase would be observed if any additional mechanism (based for example on translational control) exists to limit fluctuations in TIAR levels.

Exons 5A and 5B and parts of flanking introns are almost identical in the chicken and human tiar genes

The chicken exons 5A and 5B are identical in sequence to a corresponding pair of TIA-1-activated exons from the human tiar gene (see Fig. 3 in (21); note that the numbering system used for human tiar exons in (21) differs from that used for chicken tiar exons here, and so
human tiar exons 6 and 7 in (21) correspond in fact to chicken tiar exons 5 and 6). This high level of sequence conservation extended to the intron separating exons 5A and 5B, and to part of the intron sequences flanking these exons (Fig. 8A). The strong conservation of exons 5A and 5B and flanking intron sequences between chickens and humans suggests that their splicing is physiologically important. Although the intron between human tiar exons 11 and 12 (which correspond to chicken tiar exons 10 and 11) contains a TIA-1-activated exon, exon 11A (21), this exon shows no significant similarity to chicken exon 10A, nor do any other portions of the human and chicken introns (Fig. 8B).
DISCUSSION

We have isolated DT40 cells (tia-1(-/-)tiar(+/-) cells) with both tia-1 alleles and one tiar allele disrupted. We have not been able to disrupt the intact tiar allele in these cells, our attempts to do so all leading to retargeting of the already disrupted allele. However, if tia-1(-/-)tiar(+/-) cells are forced to express TIA-1 by transfection of a cDNA expression vector, targeted disruption of their remaining intact tiar allele then becomes possible. We conclude that DT40 cells require either TIA-1 or TIAR for viability. This suggests that the embryonic lethality observed following disruption of tia-1 or tiar genes in mice (7,26) is due to reduced cell viability, rather than problems with some specific process required for proper development.

The results resumed above indicate that TIA-1 and TIAR can play redundant roles in cell viability. Consistent with this, while the serum-dependence for growth and survival of tia-1(-/-) and tiar(+/+) cells is indistinguishable from that of DT40 cells, it is markedly higher in cells obtained from tia-1(-/-) cells by inactivating a tiar allele, or in cells obtained from tiar(+/+) cells by inactivating both tia-1 alleles. However, TIA-1 could be a less effective mediator of tasks required for cell viability than TIAR. The tia-1(-/-)tiar(+/+) cells expressing TIA-1 from an expression vector that were used for disruption of both tiar alleles contain more TIA-1 than DT40 cells, and it may be that disruption of both tiar alleles is only possible in DT40 cells expressing higher than normal levels of TIA-1. Thus despite our best efforts we have not been able to disrupt both tiar alleles in DT40 cells (our unpublished observations). While it is possible to obtain mouse cells lacking TIAR (7), the cells obtained express more TIA-1 than do wild-type cells (10). The notion that TIA-1 is less effective than TIAR is also consistent with the lower embryonic lethality observed with mice lacking TIA-1 as compared to mice lacking TIAR (7,26).
Depletion of TIA-1/TIAR leads to cell death, but so does overexpression of these proteins. Although the effects of changing TIA-1/TIAR levels on cell viability could be the consequence of problems with either translational control or splicing control, an explanation based on splicing control is particularly easy to envisage. Furthermore, such an explanation would be consistent with observations that several genes involved in apoptosis can code for both pro-apoptotic and anti-apoptotic proteins, depending on the alternative splicing choice made when splicing their pre-mRNAs (38). Clearly TIA-1/TIAR depletion could significantly reduce splicing of some exons (as observed here for tiar exon 5A), and this could lead to cell death (see exon 2 in Fig. 9). On the other hand, TIA-1/TIAR overexpression could increase splicing of some other exons (as it does for tiar exons 5A and 5B), and this could also lead to cell death (see exon B in Fig. 9).

So should TIA-1 and TIAR be looked upon as apoptosis-promoting proteins (17,20), or as proteins required for cell survival, which are inactivated during apoptosis? Upon Fas induction of apoptosis, TIA-1 becomes phosphorylated (24), and TIAR is relocated to the cytoplasm (25). It could be that TIA-1 is activated by phosphorylation for participation in events necessary for apoptosis progression. On the other hand, it is possible that TIA-1 phosphorylation is one facet of a general mechanism for splicing inhibition during apoptosis by inactivation of splicing factors (39-41). Similarly, TIAR translocation to the cytoplasm could actively promote apoptosis by bringing it into contact with cytoplasmic substrates (25), but it is equally possible that reducing nuclear TIAR levels is another facet of this general mechanism for splicing inhibition. The observations that overexpression of TIA-1 or TIAR can lead to apoptosis are difficult to interpret, as there is no evidence that such overexpression occurs normally. In addition, it is possible that overexpression of many splicing factors necessary for cell viability (such as ASF/SF2, for example (42)) will perturb splicing sufficiently to induce apoptosis. Interestingly, it has recently been shown that recombinant Sendai viruses expressing a short transcript which binds TIAR are non-cytopathic (23). Apoptosis triggered by 20-hydroxyecdysone during pupal metamorphosis of the silkworm Bombyx mori is accompanied
by an increase in TIAR mRNA levels (43). Ischemia-induced apoptosis also leads to an increase in TIAR mRNA levels (44,45). These observations suggest that TIAR could be required for apoptosis. Maybe TIA-1 and TIAR normally work for cell survival, but during apoptosis they are modified, so as to be rendered inactive for cell survival tasks, yet active to participate in new tasks in apoptosis progression. One possible way of achieving this could be by providing a new interacting partner for them.

All previous work implicating TIA-1/TIAR in splicing control has involved in vitro studies, or transient transfection of exogenous TIA-1/TIAR expression vectors or reporter genes (16,17,21). However, definitive evidence for the involvement of TIA-1/TIAR in splicing control required a demonstration that reducing the levels of endogenous TIA-1/TIAR alters the splicing of an endogenous pre-mRNA. The chicken lines with disrupted tia-1 and tiar genes have now allowed us to provide such evidence: reducing the levels of endogenous TIA-1/TIAR reduces the splicing of tiar alternative exon 5A on the endogenous tiar pre-mRNA. Splicing of the alternative 5A exon decreases in cells with both tia-1 alleles disrupted, and a greater decrease is seen in cells with one tiar allele inactivated. In cells with both tia-1 alleles and one tiar allele disrupted, splicing of the exon drops to very low levels. These results underscore the overlapping functions of TIA-1 and TIAR in splicing activation.

Exon 5A together with exon 5B make up a pair of chicken tiar alternative exons with in-frame stop codons whose splicing increases markedly following TIA-1 overexpression. The results reported here extend significantly our previous observation (21) that transient overexpression of TIA-1 activates splicing of alternative human tiar exons with stop codons, as we now show that stable TIA-1 overexpression blocks production of TIAR protein, and that this involves two phenomena. In addition to blocking TIAR production by introducing premature stop codons into the TIAR mRNA, inclusion of the alternative exons appears to expose mRNAs to nonsense-mediated mRNA decay (37): the abundance of mRNAs with the exons included rises significantly in cells treated with cycloheximide.
Negative feedback regulation of splicing as we have observed for the tiar gene appears to be a common mechanism controlling the level of splicing regulators, with for example the SR protein SC35 autoregulating its expression by promoting splicing events affecting the 3'UTR of its mRNAs and which expose them to nonsense-mediated mRNA decay (46). The extremely high sequence conservation between the human and chicken tiar genes of the stop codon-containing alternative tiar exons, and indeed of parts of the introns flanking them, is in favour of physiological importance for the negative feedback regulation of tiar alternative exon splicing. Clearly, TIA-1/TIAR-induced splicing of stop codon-containing exons could be used to stop TIA-1/TIAR levels from rising to high levels. This could be important, as high levels of these proteins can induce apoptosis. On the other hand, if TIA-1/TIAR levels drop below normal levels, this could be deleterious for cell viability. However, splicing of stop codon-containing exons should fall as a result, and this could lead to a rise in TIAR mRNA and protein levels. Finally, it is tempting to wonder if controlling splicing of stop codon-containing exons is the only mechanism used to limit fluctuations of TIAR levels: could TIA-1 and TIAR, proteins known to be involved in translational control (5-15), perhaps repress translation of their own mRNAs?

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FIGURE LEGENDS

Figure 1. Schematic representations of tia-1 and tiar genes and proteins. A. The exon-intron structure of the genes is shown, with exons marked as black boxes, and introns as lines. We have not determined the size of the intron between tiar exons 1 and 2, and so this intron is marked by a broken line. B. The structures of TIA-1 and TIAR are shown, with their three RNA recognition motifs (RRM-1 to 3) and glutamine-rich domains (Q-Rich). For RRM-2, the two RNP motifs are represented by black boxes. The parts of TIA-1 and TIAR encoded by exons 5 and 6 (ex 5 and ex 6) are shown, as is the position within exon 5 where drug-resistance markers were inserted (insert).

Figure 2. Tia-1 disruption. A. Schematic representations of the tia-1 targeting vector, an intact tia-1 allele, and the disrupted tia-1 allele obtained by targeting of the intact allele. Drawings are not to scale. Exons are represented by numbered boxes. Neo/puro represent 2.3 kb DNA fragments corresponding to neomycin- or puromycin-resistance cassettes. The origins of Eco RV fragments visualized in B are shown. B. Southern blot. DNA from cells as indicated was digested by EcoRV, migrated on an agarose gel and transferred to a nylon filter before hybridization with a probe covering tia-1 exons 6-10 derived from the TIA-1 cDNA. Tia-1(+/−): cells with one tia-1 allele disrupted. Tia-1(−/−): cells with both tia-1 alleles disrupted. C. Western blot. Proteins from cells as indicated (clone 3-1+TIA-1 is a clone with both tia-1 alleles, and one tiar allele disrupted, and which expresses TIA-1 from an integrated expression vector) were migrated on a 10% SDS-polyacrylamide gel before transfer to a nitrocellulose filter and analysis with antibodies against TIA-1. Protein samples were also analysed with antibodies against ASF/SF2 as a control.
Figure 3. Disruption of a tiar allele with targeting vector 1. A. Schematic representations of the tiar targeting vector, intact tiar alleles 1 and 2, and disrupted tiar alleles obtained by targeting of the intact alleles. Drawings are not to scale. Exons are represented by numbered boxes. Hygro represents a 3.3 kb DNA fragment corresponding to a hygromycin B-resistance cassette. The origins of Eco RV fragments visualized in B are shown. In clone 3-1 tiar allele 1 is disrupted, and in clone 3-2 tiar allele 2 is disrupted as shown. B. Southern blot. DNA from cells as indicated was digested by EcoRV, migrated on an agarose gel and transferred to a nylon filter before hybridization with a probe covering tiar exons 3-5 derived from a tiar cosmid. Tiar(1+/2-): cells with tiar allele 2 disrupted. Clone 3-1: cells with both tia-1 alleles, and tiar allele 1 disrupted. Clone 3-2: cells with both tia-1 alleles, and tiar allele 2 disrupted. The weak band marked by an asterisk in lane 1 is an overdigestion product. C. Western blot. Proteins from cells as indicated were migrated on a 10% SDS-polyacrylamide gel before transfer to a nitrocellulose filter and analysis with antibodies against TIAR. Protein samples were also analysed with antibodies against ASF/SF2 as a control.

Figure 4. Tiar allele disruption with targeting vector 2. A. Schematic representations of tiar targeting vector 2, intact tiar alleles 1 and 2, hygro disrupted alleles 1 and 2 as in Fig. 3, and disrupted tiar alleles obtained following targeting by vector 2 of the intact alleles or the hygro disrupted alleles. Drawings are not to scale. Exons are represented by numbered boxes. hygro represents a 3.3 kb DNA fragment corresponding to a hygromycin B-resistance cassette, while gpt represents a mycophenolic acid-resistance cassette. 3'ss and pA are a 3' splice site sequence and a polyadenylation signal, respectively. The origins of Eco RV fragments visualized in B are shown. B, C. Southern blots. DNA from cells as indicated was digested by EcoRV, migrated on an agarose gel and transferred to a nylon filter before hybridization with a probe covering tiar exon 2 and 2 kb of downstream intron. Clone 3-1 and clone 3-2 are as in...
the legend to Fig. 3. Clone 3-1*: clone 3-1 cells with the hygro-disrupted allele 1 targeted a second time. Clone 3-2*: clone 3-2 cells with the hygro-disrupted allele 2 targeted a second time. Clone 4+TIA-1: clone 3-1 cells with both tia-1 and both tiar alleles disrupted, but which express TIA-1 from an integrated expression vector. The clone 4+TIA-1 sample was run on the same gel as the two other samples, but not on an adjacent lane on the gel. The three samples have been regrouped to save space here. D. Western blot. Proteins from cells as indicated were migrated on a 10% SDS-polyacrylamide gel before transfer to a nitrocellulose filter and analysis with antibodies against TIAR or TIA-1. Clone 3-1+TIA-1 and clone 3-1*+TIA-1 are respectively clone 3-1 and clone 3-1* cells which express TIA-1 from an integrated expression vector. For the TIA-1 blot, all samples were run on the same gel, but samples for lanes 1-3 and lanes 4-5 were not on adjacent lanes on the gel. They have been regrouped to save space here.

Figure 5. Growth curves. Cells were inoculated into medium containing 1% chicken serum, and 0%, 1% or 10% fetal calf serum as shown, and the viable cell count determined after various times of incubation. Each experiment was carried out at least three times, and typical results are shown.

Figure 6. TIA-1 overexpression leads to splicing of tiar alternative exons. A. RNA from clone 3-1 and clone 3-1 expressing TIA-1 from an integrated expression vector (clone 3-1+TIA-1) was used for RT-PCR analysis with primers in tiar exons 1 and 5 (lanes 1 and 2), or in exons 5 and 8 (lanes 3 and 4), or in exons 7 and 12 (lanes 5 and 6). PCR products were migrated on an agarose gel and transferred to a nylon filter before hybridization with a tiar cDNA probe. Bands marked a, b and c appearing in clone 3-1+TIA-1 only are identified, and their structures are represented schematically. Note that the results shown for lane 4 result from a 12-fold
longer exposure of the film than that used for the other lanes. B. Sequences of alternative tiar exons 5A, 5B and 10A activated by TIA-1. Exon sequences are marked in capital letters. Stop codons in the reading frame of the upstream tiar exon are marked by asterisks.

Figure 7. Tiar alternative exon splicing drops when TIA-1/TIAR is depleted. A. RNA from cells as marked which had been incubated alone or in the presence of cycloheximide (50 µg/ml) for 2 h was used for RT-PCR analysis using primers in tiar exons 5 and 8. PCR products were migrated on an agarose gel and transferred to a nylon filter before hybridization with a tiar cDNA probe. Bands corresponding to splicing of exon 5A alone, or exon 5A and 5B together are identified (their identities were established by sequencing). B, C. Quantitative PCR. Each bar represents the mean with S.D. of the –fold increase of the sample over that of the DT40 sample. Samples of cDNA from cells as shown were subjected to quantitative PCR using for B primers in exons 5 and 5A so as to quantify together cDNAs with either exon 5A or exon 5A spliced to exon 5B or for C a primer in exon 5 and a primer crossing the exon 5/exon 6 junction, to quantify cDNAs corresponding to normal TIAR mRNA without exon 5A or exon 5B. For B, results were compared to the level of exon 5A splicing in DT40 cells not exposed to cycloheximide, to which the value 1 was arbitrarily attributed. For B, results shown represent the levels of exon 5A-containing mRNAs or TIAR mRNAs obtained from one tiar allele, i.e. we have corrected for the fact that some lines have one tiar allele, while others have two. For C, results were compared to the level of exon 5 to exon 6 splicing in DT40 cells not exposed to cycloheximide, to which the value 1 was arbitrarily attributed. Determinations were repeated four times, and mean values are shown.

Figure 8. Comparison of human and chicken tiar genes. A. Comparison of the human tiar exon 6-7 region containing the TIA-1 activated alternative 6A and 6B exons, to the corresponding
region of the chicken gene containing the TIA-1 activated alternative 5A and 5B exons. Note that the human exons 6 and 7 correspond to the chicken exons 5 and 6. Regions where the sequence homology is superior to 95% are shown by heavy black lines. B. Comparison of the human tiar exon 11-12 region containing the TIA-1 activated alternative 11A exon, to the corresponding region of the chicken gene containing the TIA-1 activated alternative 10A exon. Note that the human exons 11 and 12 correspond to the chicken exons 10 and 11. Regions where the sequence homology is superior to 85% are shown by heavy black lines.

Figure 9. Hypothesis for the effect of changing TIA-1/TIAR levels on cell viability. For the gene with exons 1, 2 and 3, splicing of exon 2 is required for viability. Depletion of TIA-1/TIAR prevents splicing of this exon, and leads to cell death. For the gene with exons A, B and C, splicing of exon A to exon C is required for viability. Overexpression of TIA-1/TIAR activates splicing of exon B, and leads to cell death.
Figure 1

A.

\[ \text{tia-1:} \quad \begin{array}{ccccccccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 & 11 & 12 & 1 \text{ kb}
\end{array} \]

\[ \text{tiar:} \quad \begin{array}{ccccccccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 & 11 & 12
\end{array} \]

B. TIA-1/TIAR:

\[ \begin{array}{cccc}
\text{RRM-1} & \text{RRM-2} & \text{RRM-3} & \text{Q-Rich}
\end{array} \]

\[ \begin{array}{cccc}
\text{RNP-2} & \text{RNP-1}
\end{array} \]

ex 5 ex 6

insert
Figure 2

A.

Targeting vector: 

| 1 | 2 | 3 | 4 | 5 | neo/puro | 6 | 7 | 8 | 9 | 10 |

Intact allele: 

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |

Disrupted allele: 

| 1 | 2 | 3 | 4 | 5 | neo/puro | 6 | 7 | 8 | 9 | 10 | 11 | 12 |

B.

DT40  tia-1(+/−)  tia-1(−/−)

7.8 kb Disrupted

5.5 kb Intact

1 2 3 4 5

C.

DT40  tia-1(+/−)  tia-1(−/−)  clone 3-1+TIA-1

TIA-1

ASF/SF2

1 2 3 4 5 6

kDa

50

37

25
Figure 3

A.

Targeting vector 1:

Intact allele

Disrupted allele

Clone 3-1

Clone 3-2

B.

Intact allele 1
(12 kb)

Intact allele 2
(7.6 kb)

C.

Disrupted
allele 1 (15.3 kb)

Disrupted
allele 2 (10.9 kb)

TIAR

ASF/SF2
Figure 4

A.

**gpt disrupted allele**

1. EcoRV
2. EcoRV
3. EcoRV
4. EcoRV
5. EcoRV

11.5 kb

**Intact allele**

1. EcoRV
2. EcoRV

12 kb

**TARGETING VECTOR 2:**

1. EcoRV
2. EcoRV
3. EcoRV
4. EcoRV
5. EcoRV

7.6 kb

**hygro disrupted allele**

1. EcoRV
2. EcoRV
3. EcoRV
4. EcoRV
5. EcoRV

15.3 kb

**hygro+gpt disrupted allele**

1. EcoRV
2. EcoRV
3. EcoRV
4. EcoRV
5. EcoRV

11.5 kb
Figure 4

B.

Intact allele 1 (12 kb)

Hygro disrupted allele 2
(10.9 kb)

Hygro disrupted allele 2
(7.1 kb)

C.

hygro disrupted allele 1
(15.3 kb)

hygro+gpt disrupted allele 1
(11.5 kb)

Intact allele 2
(7.6 kb)

D.

DT40 clone 3-1
clone 3-1+TIA-1
clone 3-1*+TIA-1
clone 4+TIA-1

TIAR

TIA-1

kDa

50

37

25

1 2 3 4 5

1 2
Figure 5

A. 3-1 TIA-1

B. clone 3-2 clone 3-1 clone 3-1+TIA-1

Viable cells/ml

10% FCS

DT40 tia-1(-/-) tiar(+/-)

1% FCS

0% FCS

Viable cells/ml

Time (hours)

10% FCS

DT40 tia-1(-/-) tiar(+/-)

Viable cells/ml

Time (hours)

1% FCS

0% FCS

Viable cells/ml

Time (hours)

10% FCS

DT40 tia-1(-/-) tiar(+/-)

Viable cells/ml

Time (hours)

1% FCS

0% FCS

Viable cells/ml

Time (hours)

10% FCS

DT40 tia-1(-/-) tiar(+/-)

Viable cells/ml

Time (hours)

1% FCS

0% FCS

Viable cells/ml

Time (hours)
**Figure 6**

A. 

| ex 1-5 | ex 5-8 | ex 7-12 |
|--------|--------|---------|
| clone 3-1+TIA-1 | clone 3-1 | clone 3-1+TIA-1 |

B. 

5A: 

```
attttcagAATTGCTCATAAGAATGGACAGGATCTTGAAGGCTAACTGCAAGGAACTGTGCACACTGGAACTCAGTTgtagattttttttctcatttctattatatcttattatacattattatatatatacatattttagtatttacatttttaaca
```

10A: 

```
ctgtctcagTTATGTTCCAGTTGTTTCATCAAATTGGGCTAATGATTTTGGTGGAATATCTGTCTTCTGGATATGATTCAAGATAGGGAGAAACTTGGAGgtaattctgattcttgctagcattcttactgcagactagctgactgtccttccaaa
```

5B: 

```
aatttacagCTGCCCAAACTCCAAAATGATGGTAGAAGAATTGACCAAGAAAAA
```

10A: 

```
AATATCTGTCTTCTGGATATGATTCAAGATAGGGAGAAACTTGGAGgtaattctgattcttgctagcattcttactgcagactagctgactgtccttccaaa
```
Figure 7

A.

Relative amount of tiar mRNA

DT40 clone 3-1 clone 3-1+TIA-1

- Cycloheximide

+ Cycloheximide

B.

Relative exon 5A use/tiar allele

DT40 tia-1(-/-) tia(-/-/+) clone 3-1 clone 3-2

- Cycloheximide

+ Cycloheximide

C.

Relative amount of tiar mRNA

DT40 clone 3-1 clone 3-1+TIA-1

- Cycloheximide

+ Cycloheximide
Figure 8

A.

Human tiar

Homology > 95%

Chicken tiar

B.

Human tiar

Homology > 85%

Chicken tiar

200 bp
Figure 9

Survival:

1 2 3

Depletion

1 2 3

Overexpression

A B C

Death:

1 3

A B C
TIA-1 or TIAR is required for DT40 cell viability
Caroline Le Guiner, Marie-Claude Gesnel and Richard Breathnach

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