

Comparative analysis of DNA vectors at mediating RNAi in *Anopheles* mosquito cells and larvae

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Summary

Heritable RNA interference (RNAi) mediated by transgenes exhibiting dyad symmetry represents an important tool to study the function of genes expressed at late developmental stages. In this study, we determined whether the transcriptional machinery of *Anopheles* mosquitoes is capable of directing suppression of gene expression from DNA constructs designed to transcribe double-stranded RNA (dsRNA) as extended hairpin-loop RNAs. A series of DNA vectors containing sense and antisense regions of the green fluorescent protein *EGFP* target gene was developed. The effect of these vectors on a transiently expressed or stably integrated *EGFP* gene

was assessed in an *Anopheles gambiae* cell line and in *Anopheles stephensi* larvae. Our data indicate that dsRNA-mediated silencing of a target gene from plasmid DNA can be achieved at high levels in *Anopheles* cell lines and larvae. The region that links the sense and antisense sequences of the target gene plays a determining role in the degree of silencing observed. These results provide important information for the development of heritable RNAi in *Anopheles*.

Key words: RNAi, heritable RNA interference, *Anopheles*, mosquito, *EGFP*, DNA vector, gene silencing.

Introduction

Transmission of human malaria, a disease that causes more than one million deaths a year, is accomplished by a small group of mosquito species belonging to the *Anopheles* genus. The recent development of an efficient gene transfer technology for *Anopheles stephensi* and *Anopheles gambiae* mosquitoes (Catteruccia et al., 2000a; Grossman et al., 2001), combined with the recent completion of the *A. gambiae* genome sequence project (Holt et al., 2002), has paved the way for studies on the genetics of these important disease vectors. However, progress towards the identification of mosquito molecules involved in the interaction with the *Plasmodium* malaria parasite has been hampered by the lack of efficient technologies, such as mutagenesis screens and gene knock-out by homologous recombination, routinely used in other organisms to perform functional genomic studies. Gene silencing by RNA interference (RNAi), an evolutionarily conserved phenomenon triggered by double-stranded RNA molecules (dsRNAs), has recently been envisaged as a powerful tool for studying gene function in different model organisms (Fire et al., 1998). Although the mechanism by which dsRNA mediates gene silencing is not fully understood, it is known to invoke a multi-step process that results in the cleavage of long dsRNAs into 21–25 nucleotide fragments that then direct the sequence-specific degradation of homologous endogenous mRNA (Bernstein et al., 2001; Elbashir et al., 2001; Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000).

To date, the only reported attempts at using RNAi to study gene function in *Anopheles* mosquitoes have relied upon the direct delivery of high concentrations of *in vitro*-synthesised dsRNA molecules in cell lines and adults (Blandin et al., 2002; Levashina et al., 2001). While this approach offers a rapid genetic screen of putative target genes, interference to gene expression is transient, non-inheritable and subject to significant variations between individuals. In some organisms, including *Caenorhabditis elegans* (Tavernarakis et al., 2000), trypanosomes (Bastin et al., 2000; Shi et al., 2000), *Drosophila* (Fortier and Belote, 2000; Kennerdell and Carthew, 2000; Piccin et al., 2001) and plants (Smith et al., 2000), stable expression of dsRNA from integrated transgenes exhibiting dyad symmetry has recently been achieved, thus overcoming the problems related to the transient nature of RNAi mediated by the delivery of *in vitro*-synthesised dsRNA. The stable RNAi approach is anticipated to contribute significantly to the investigation of the function of genes involved in mosquito–parasite interactions in *Anopheles* mosquitoes.

In this study, we have assessed the molecular requirements for maximising the silencing efficiency of dsRNA-encoding genes in *Anopheles*. We have developed a series of constructs marked with the red fluorescent protein DsRed (Matz et al., 1999), in which sense and antisense regions of the coding sequence of the green fluorescent protein *EGFP* (Heim and Tsien, 1996) were connected by different spacers and placed

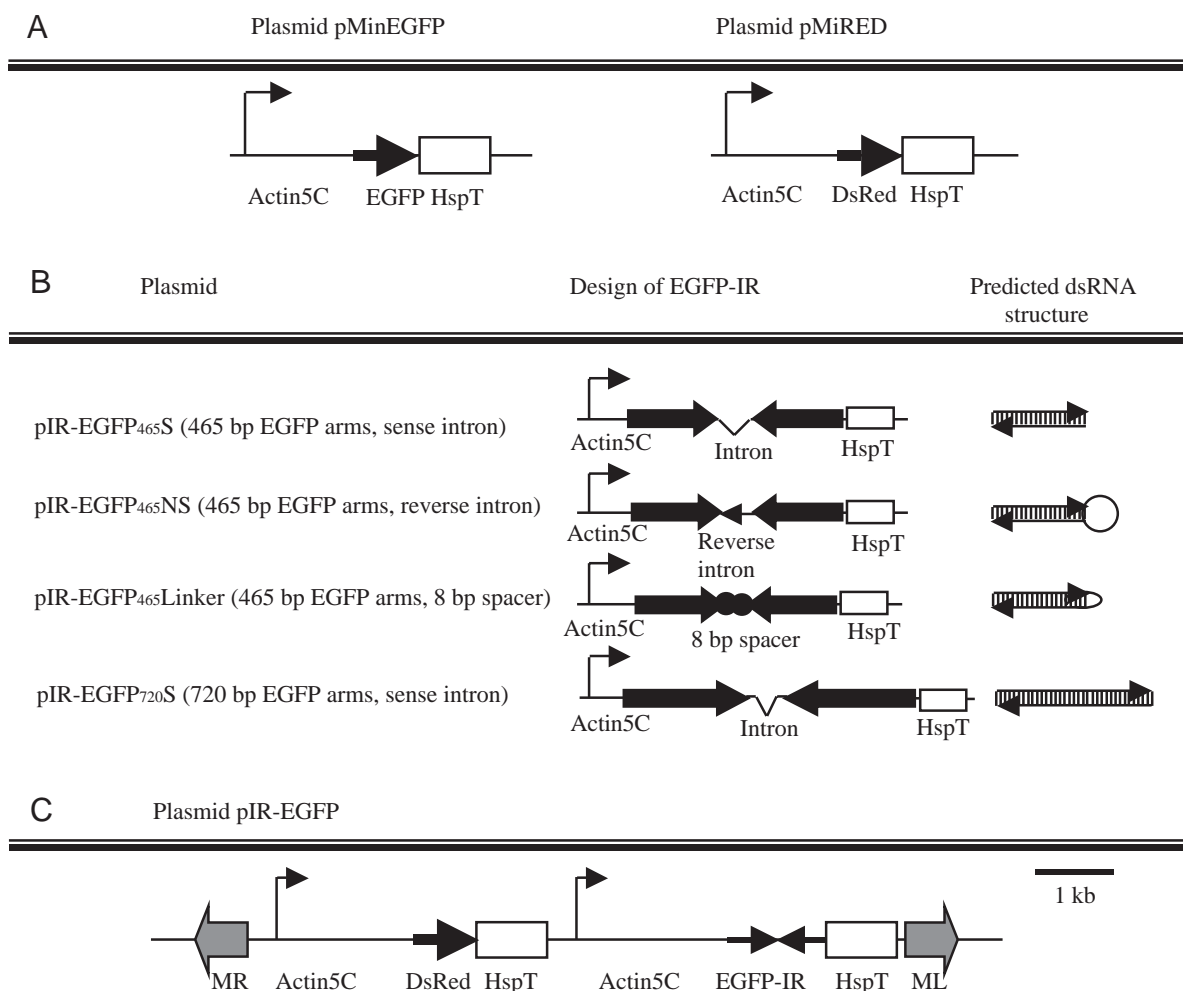


Fig. 1. Plasmid maps and predicted structure of dsRNA transcripts. (A) Schematic representation of target plasmid pMinEGFP and control plasmid pMiRED. (B) Design of EGFP inverted repeats (EGFP-IR) and predicted structure of dsRNA transcript. Filled arrows pointing in opposite directions represent the EGFP-IR. Spacer regions are shown. (C) Schematic representation of generic dsRNA-expression plasmid, pIR-EGFP. Each IR-EGFP construct was cloned into pMiRED within the arms of *minos*. Actin5C, *D. melanogaster actin5C* promoter; HspT, *D. melanogaster Hsp70* terminator sequence. The EGFP-, EGFP-IR- and DsRed-encoding genes are indicated by filled arrows. ML, *minos* left arm; MR, *minos* right arm.

under the control of a constitutive promoter. The silencing activity of these constructs was assessed in *A. stephensi* larvae and *A. gambiae* cells transiently expressing the *EGFP* target gene. Furthermore, an RNAi construct was also analysed for its ability to silence an *EGFP* gene stably integrated in the genome of *A. stephensi* larvae.

Materials and methods

Plasmid construction

All EGFP inverted repeats (IR) were produced by duplicating, with opposite direction, the *EGFP* gene (Clontech, Palo Alto, USA) by directionally inserting a 465 bp (from position 219 to 683) or full-length 720 bp (from 1 to 719) PCR product into the plasmid pCR2.1v (Invitrogen, Paisley, UK). *Bam*HI and *Eco*RV restriction sites were added to the 5' and 3' ends of the sense arm product, respectively, and *Xba*I and *Xho*I

restriction sites to the 5' and 3' ends of the antisense arm, respectively. To prepare pIR-EGFP₄₆₅S and pIR-EGFP₇₂₀S, a 67 bp PCR product corresponding to intron 1 from *A. gambiae* lysozyme gene (Kang et al., 1996; GenBank accession U28809) was amplified from *A. gambiae* genomic DNA, adding *Eco*RV and *Xho*I restriction sites to the 5' and 3' ends, respectively, and inserted between the EGFP-IR (Fig. 1B). pIR-EGFP₄₆₅NS was produced by inserting the intron described above in its reverse non-splicing orientation, by inverting the restriction sites (Fig. 1B). pIR-EGFP₄₆₅Linker was produced by inserting a non-palindromic 10 bp oligonucleotide (ATCGTTAACC) between the EGFP-IR (Fig. 1B). In each construct, the IR-EGFP cassette was then inserted between *actin5C* promoter and *Hsp70* terminator elements from *Drosophila melanogaster*, and cloned as a *Not*I cassette into pMiRED (Nolan et al., 2002), which contained the *DsRed* marker gene also cloned under the control of the *actin5C* promoter (Fig. 1A,C).

Cell culture and plasmid transfections

A. gambiae Sua 4.0 cells were grown in Schneider's *Drosophila* medium (GIBCO, Paisley, UK) supplemented with 10% foetal calf serum (FCS), 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Invitrogen) at 25°C. A total of 4×10⁵ cells ml⁻¹ were plated 24 h before co-transfection experiments, and solutions containing 4 µg each of pMinEGFP (Fig. 1A) and either pMiRED or a dsRNA-transcribing plasmid were co-transfected as described previously (Catteruccia et al., 2000b). Cells were examined two days after transfection at a wavelength of 490 nm to detect EGFP expression and 565 nm to detect DsRed expression.

Mosquito breeding and rearing

Wild-type and transgenic *Anopheles stephensi* Liston adult mosquitoes (strain sd 500) were maintained at 28°C, 70% humidity and fed on 5% glucose. To induce egg production, female mosquitoes 3–5 days old were starved for 5 h and allowed to feed on mouse blood. Two days after blood feeding, eggs were laid and transferred into buckets with H₂O containing 5% NaCl. Larvae were grown at 25°C, 70% humidity and fed on fish food. After 10–12 days, pupae were collected and adult mosquitoes were allowed to emerge in cages.

Embryo microinjection

A. stephensi mosquito embryos were injected essentially as described previously (Catteruccia et al., 2000a). For transient RNAi studies, wild-type *A. stephensi* embryos were injected with a mixture of pMinEGFP (400 µg ml⁻¹) and either pMiRED or one of the dsRNA-transcribing plasmids (400 µg ml⁻¹). For RNAi studies against stably expressed EGFP, *A. stephensi* line V_B (Catteruccia et al., 2000a) was injected with plasmid pIR-EGFP₄₆₅Linker (400 µg ml⁻¹). The levels of EGFP expression were assessed daily in larvae positive for DsRed at the wavelengths described above.

Quantitative analysis of RNAi

Cells and larvae were captured on a Nikon inverted microscope with an attached Nikon DXM1200 digital camera. Fluorescent gene expression was quantified using the Lucia G image-processing and analysis software (Version 4.61, Nikon UK, Kingston, UK). The levels of EGFP and DsRed expression were measured using the MeanGreen and MeanRed feature of the software, which calculates the statistical mean of the intensity of the green or red components of pixels, respectively. Using this software, it was possible to calculate the mean EGFP fluorescence in only those cells in which DsRed was co-expressed. The software was validated by measuring the intensity of EGFP expression in homozygous and heterozygous larvae from the *A. stephensi* line V_B. Briefly, individuals homozygous for the *EGFP* insertion consistently showed MeanGreen values that were approximately double those from heterozygous larvae of the same age, demonstrating a good correlation between MeanGreen values and transgene copy number. For statistical analyses, unpaired *t*-tests were performed; the null hypothesis was rejected at *P*≥0.05.

Results

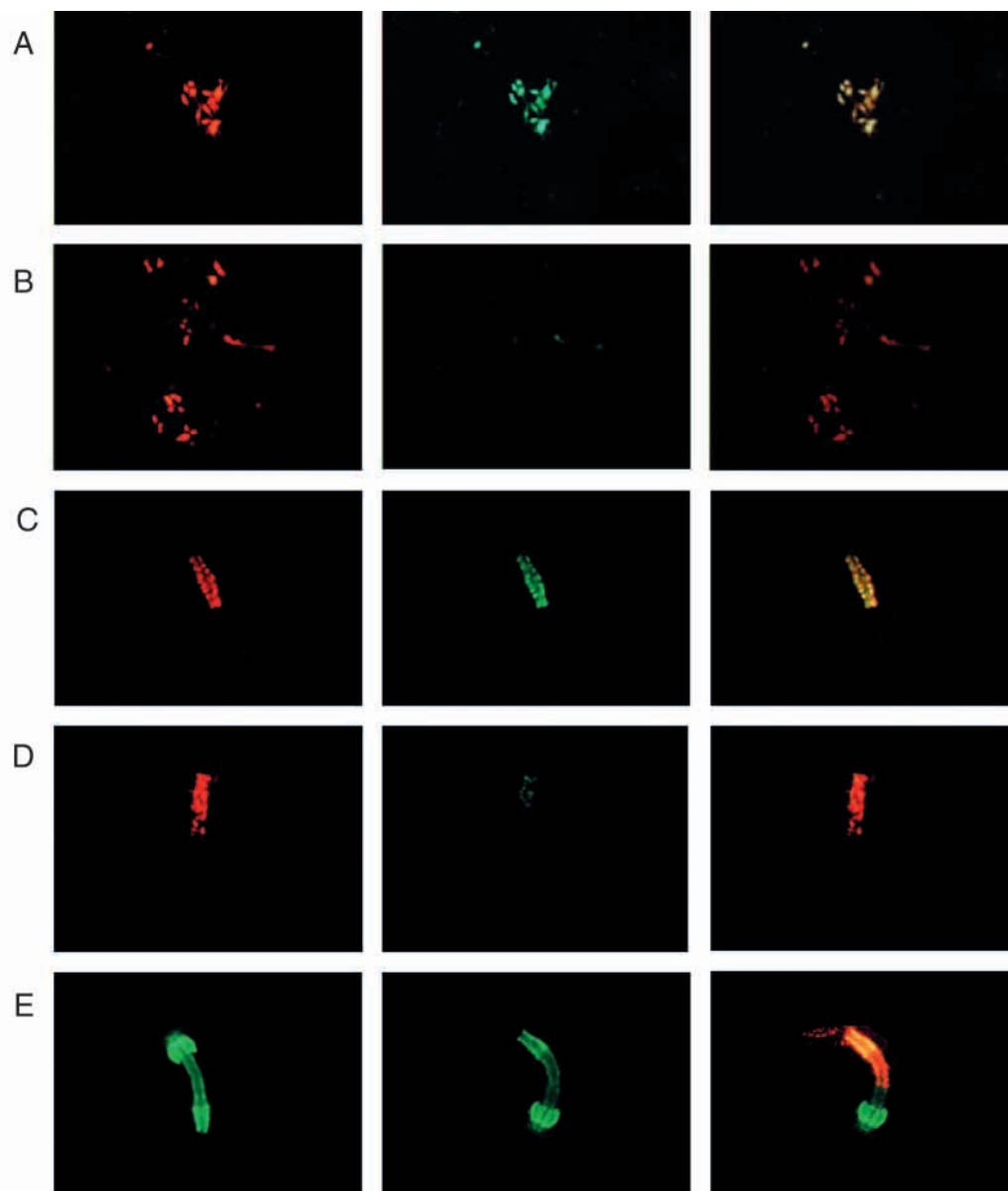
Design of dsRNA-expressing plasmids

In order to create a reliable and objective model of RNAi in *Anopheles*, the green fluorescent protein *EGFP* from plasmid pMinEGFP (Catteruccia et al., 2000a; Fig. 1A) was selected as the target gene, since it allowed a direct and non-invasive investigation of dsRNA-mediated silencing at different time points without the need to sacrifice individuals. Studies in plants have suggested that the region linking the perfect inverted repeat (IR) sequences of the target gene is critical to silencing efficiency (Smith et al., 2000). In this light, a series of DNA constructs encoding dsRNA molecules were developed, containing sequences of the *EGFP* target gene cloned in sense and antisense orientations and linked in a head-to-head manner by distinct spacer regions. In the tobacco plant *Nicotiana tabacum*, the process of intron excision from hairpin RNA (hpRNA) has been reported to enhance gene-silencing efficiency (Smith et al., 2000). To assess whether this phenomenon could also be observed in *Anopheles*, a construct was developed that contained 465 bp sense and antisense *EGFP* sequences flanking a 67 bp intron from the *A. gambiae* lysozyme gene (Kang et al., 1996; pIR-EGFP₄₆₅S; Fig. 1B). The same intron was also cloned in its inverted non-splicing orientation in construct pIR-EGFP₄₆₅NS, with the aim of producing a long hairpin loop in the dsRNA molecule (Fig. 1B). Furthermore, to determine whether the length of the hairpin loop affects silencing efficiency, construct pIR-EGFP₄₆₅Linker was developed, which contained a 10 bp-long linker between the sense and antisense *EGFP* arms (Fig. 1B). An additional construct, pIR-EGFP₇₂₀S, contained 720 bp arms corresponding to the full length of the *EGFP* gene separated by the *A. gambiae* intron in its splicing orientation (Fig. 1B), as studies in other organisms have shown that the length of the dsRNA affects RNAi efficiency (Ngo et al., 1998; Parrish et al., 2000; Tuschl et al., 1999). All dsRNA constructs were derived from plasmid pMiRED (Nolan et al., 2002), which contained a red fluorescent protein (*DsRed*) marker gene cassette inserted within the arms of the *minos* transposable element (Franz and Savakis, 1991), with the perspective of developing stable transgenic lines expressing dsRNA (Fig. 1A). The *EGFP* target gene from plasmid pMinEGFP (Fig. 1A) and the dsRNA-transcribing genes from each IR construct were all placed under the control of the *actin5C* promoter from *Drosophila melanogaster*, to ensure similar transcriptional levels. A schematic representation of all dsRNA constructs is given in Fig. 1C. All IR constructs were *in vitro* transcribed to prove they could produce dsRNA species (data not shown).

dsRNA-mediated gene silencing in *A. gambiae* Sua 4.0 cells

To assess the ability of a DNA-based system to mediate RNAi in *Anopheles* cells, dsRNA-transcribing constructs, marked with the *DsRed* marker gene, were co-transfected with target plasmid pMinEGFP into the *A. gambiae* Sua 4.0 cell line in a series of consecutive experiments. In this experimental system, the occurrence of RNAi against the

Fig. 2. Fluorescence microphotographs of *A. gambiae* Sua 4.0 cells and *A. stephensi* larvae. In A–D, column 1 shows DsRed fluorescence, column 2 shows EGFP fluorescence and column 3 shows DsRed fields superimposed upon EGFP fields to show co-localisation (yellow). (A) Sua 4.0 cells transfected with pMinEGFP and pMiRED show perfect co-localisation. (B) Sua 4.0 cells transfected with pMinEGFP and pIR-EGFP₄₆₅-Linker show virtually no EGFP expression. (C) Wild-type *A. stephensi* larvae injected with pMinEGFP and pMiRED show exact co-localisation. (D) Wild-type *A. stephensi* larvae injected with pMinEGFP and pIR-EGFP₄₆₅-Linker show very limited EGFP expression. Cells were captured on a Nikon inverted microscope at 20× magnification 48 h post-transfection. Larvae were photographed at 10× magnification 48 h post-hatching. (E) Four-day-old EGFP-expressing *A. stephensi* larvae from transgenic line V_B injected with pMiRED (column 1) or pIR-EGFP₄₆₅-Linker (column 2). Column 3 shows localisation of DsRed expression from pIR-EGFP₄₆₅-Linker in the same larva.



target gene will be indicated by a decrease in the intensity of EGFP fluorescence in those cells that express the DsRed marker. Control experiments were performed by co-transfecting the target construct pMinEGFP with plasmid pMiRED, containing the DsRed marker cassette but not the EGFP-IR (Fig. 1A). In these cells, perfect co-localization of the green-fluorescent target and red-fluorescent control plasmids was observed (Fig. 2A). The intensity of fluorescence of the EGFP and DsRed proteins was not affected by the expression of the other marker (data not shown). In all samples, quantitative analysis was performed using an image-processing software that allowed the quantification of the mean intensity of green and red fluorescence of each cell. In cells co-transfected with pMinEGFP and pIR-EGFP₄₆₅S, in which the *A. gambiae* intron was placed in its splicing orientation, a 93.6%

reduction of EGFP expression was observed as compared with control experiments (Fig. 3A). Cells transfected with pIR-EGFP₄₆₅NS, containing the intron in its inverted, non-splicing orientation, exhibited varying degrees of EGFP silencing in different cell subsets, ranging from almost complete to very limited silencing and averaging 70.7% with respect to controls (Fig. 3A). In cells transfected with pIR-EGFP₄₆₅Linker, in which the large hairpin loop was replaced with a short 10 bp spacer region, silencing of the target gene expression increased to 98.2% in all transfected cells (Figs 2B, 3A). High-level silencing (96.4% inhibition) was also observed when the dsRNA corresponding to the full-length EGFP sequence was delivered (pIR-EGFP₇₂₀S; Fig. 3A). In all samples analysed, dsRNA transcription did not inhibit DsRed expression, and the levels of the DsRed protein were consistently comparable to controls (Fig. 3A).

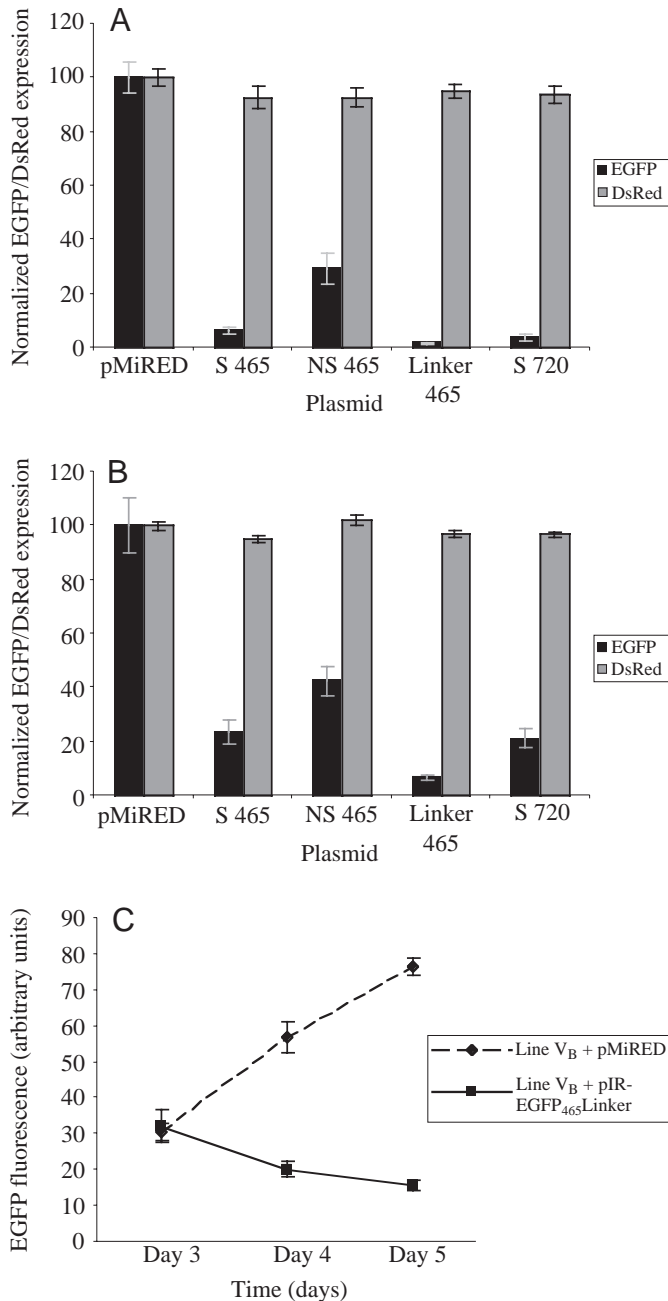


Fig. 3. Quantification of RNA interference in *A. gambiae* Sua 4.0 cells and *A. stephensi* larvae. (A) *A. gambiae* Sua 4.0 cells co-transfected with pMinEGFP and one of the dsRNA-expressing plasmids. (B) Wild-type *A. stephensi* larvae injected with pMinEGFP and one of the dsRNA-expressing plasmids. The intensity of EGFP and DsRed expression was calculated using the Lucia G image-processing and analysis software. Red and green fluorescence levels were normalised to control experiments in which pMinEGFP and pMiRED were delivered. Black bars indicate normalized EGFP expression levels; grey bars indicate normalized DsRed expression. The plotted data show mean values \pm S.E.M. from >20 fields captured in three separate transfection experiments or >20 injected larvae per experiment. All reductions in EGFP expression were found to be highly significant ($P < 0.01$). DsRed expression levels did not vary significantly between individual experiments ($P > 0.05$). S 465, pIR-EGFP₄₆₅S; NS 465, pIR-EGFP₄₆₅NS; Linker 465, pIR-EGFP₄₆₅-Linker; S720, pIR-EGFP₇₂₀S. (C) EGFP-silencing after injection of either pMiRED (broken line) or pIR-EGFP₄₆₅-Linker (solid line) into stable EGFP-expressing transgenic line V_B. The intensity of EGFP expression was calculated 3, 4 and 5 days post-hatching.

control larvae analysed, the expression of the green and red fluorescent protein largely coincided, as indicated by their overlapping profiles (Fig. 2C). However, when pIR-EGFP₄₆₅S was co-injected with the target plasmid, a 76% decrease in the level of EGFP expression was observed with respect to control larvae (Fig. 3B). A similar effect was observed after the injection of construct pIR-EGFP₇₂₀S, which caused a 79% reduction in EGFP expression (Fig. 3B). When construct pIR-EGFP₄₆₅Linker was injected, reduction of EGFP expression was almost complete, averaging 93% as compared with control larvae (Figs 2D, 3B). Although some EGFP expression was observed in larvae injected with all three constructs, it appeared to be confined to those few cells that did not exhibit expression of the *DsRed* gene. In agreement with the cell data, plasmid pIR-EGFP₄₆₅NS had a less significant silencing effect, and EGFP inhibition averaged 58% with respect to controls (Fig. 3B).

RNAi against a stably expressed EGFP target gene

We then assessed the effects of the dsRNA-transcribing construct pIR-EGFP₄₆₅Linker on a stably expressed EGFP transgene. This construct was chosen as it had consistently mediated the strongest inhibition of target gene expression in both cell lines and larvae. *A. stephensi* embryos from transgenic line V_B, stably expressing the EGFP gene under the control of the *actin5C* promoter, were injected with pIR-EGFP₄₆₅Linker, and EGFP expression was monitored throughout larval development. As a control, homozygous embryos from line V_B were also injected with plasmid pMiRED (Fig. 1A). Quantitative analysis of fluorescence was performed between three days and five days post-hatching, over which period control larvae showed a linear increase in intensity of EGFP (Fig. 3C). Injection of pIR-EGFP₄₆₅Linker significantly decreased the overall intensity of green fluorescent protein expression (Figs 2E, 3C), with the most noticeable effects observed five days post-hatching when 80%

Transient dsRNA-mediated gene silencing in *A. stephensi* larvae

To examine whether the RNAi-mediated gene silencing observed in *A. gambiae* cells could also be achieved *in vivo*, the activity of the dsRNA constructs was analysed in mosquitoes. *A. stephensi* embryos were microinjected with a mixture of the target vector pMinEGFP and one of the dsRNA-transcribing plasmids. Larvae surviving the injection procedure were analysed for DsRed fluorescence as an indicator of successful delivery of the DNA species. To ascertain the perfect co-localization of target and targeting constructs, control experiments were performed by injecting a mixture of the pMinEGFP and pMiRED plasmids. In all

silencing was achieved (Fig. 3C). Late larval stages showed more varied silencing effects. In some cases, EGFP expression was slowly restored to normal levels, while in approximately 50% of larvae gene silencing continued to be observed (data not shown). Injections were performed into the posterior end of the embryos, thus the DsRed marker was mainly localised in the last few segments of the larval abdomen (Fig. 2E).

Discussion

In this study, we performed a systematic analysis of the ability of different DNA constructs to induce RNAi in *Anopheles* cells and larvae. The advantage of using DNA constructs to generate dsRNAs is based upon their inherent stability over *in vitro*-synthesised dsRNA molecules. Furthermore, the development of an efficient heritable RNAi technique to study the function of genes expressed at late developmental stages in *Anopheles* mosquitoes will eventually rely on the use of dsRNA-transcribing constructs capable of producing high-degree silencing combined with good stability of the inverted repeats. A careful analysis of the most suitable construct is therefore particularly important at this preliminary stage to assess the molecular requirements determining the degree of gene silencing.

The results reported here show that inhibition of target gene expression depended considerably upon the spacer region separating the inverted repeats, while the length of the IR itself did not seem to significantly affect silencing efficacy. In particular, the presence of a 67 bp-long spacer (corresponding to the non-splicing orientation of the intron) dramatically reduced inhibition of EGFP expression, while the same spacer in its splicing orientation mediated high-level silencing of the target gene. While the basis for the high efficiency of gene silencing by intron-spliced dsRNA *in vivo* is not known, it is believed that the process of intron excision may transiently increase the amount of dsRNA either by promoting its formation or by creating a smaller, less nuclease-sensitive loop (Smith et al., 2000). Furthermore, the presence of an intron might increase the genomic stability of RNAi transgenes, as genomic IR have been shown to be unstable (Bi and Liu, 1996; Leach, 1994) and increasing the distance between the IR has been postulated to reduce their recombinogenic potential (Lobachev et al., 2000). On the other hand, a long spacer may obstruct the annealing of the IR, causing a modest number of dsRNA molecules to be formed, or make the dsRNA molecules more nuclease sensitive. Construct pIR-EGFP₄₆₅Linker, containing the smaller spacer, consistently mediated the highest silencing in both cells and larvae. This finding could be explained on the basis of faster kinetics of dsRNA formation, while the intron-containing constructs required splicing to occur before export to the cytoplasm where they could mediate silencing. The two intron-splicing constructs containing different IR lengths performed equally well, with no significant differences between them. This finding seems to indicate that the length of the dsRNA construct does not play a major role in silencing efficiency in *Anopheles*.

The *EGFP* target gene transiently introduced into *Anopheles* cells and embryos provided a simple and impartial model for testing the gene-silencing efficacy of the targeting constructs. This fluorescent marker allowed analysis at the protein level both visually and quantitatively using image-processing software. RNAi was also observed when a stably integrated *EGFP* gene was targeted. The analysis of RNAi against an endogenous gene could have been compromised by the occurrence of selection against constructs inducing a high degree of inhibition, as this could have had a deleterious effect on fitness and viability of the organism. The levels of *EGFP* inhibition depended on the amount of pIR-EGFP₄₆₅Linker delivered. In individuals injected with a 10-fold lower concentration of pIR-EGFP₄₆₅Linker, no significant silencing of EGFP expression was observed (not shown). Plasmid DNA persisted for many days after injection, and in most cases DsRed was still visible in fourth instar larvae. This could represent an advantage over using *in vitro*-synthesised dsRNA molecules, whose half-life has been postulated to be too limited to study late developmental genes (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999; Montgomery and Fire, 1998; Wianny and Zernicka-Goetz, 2000).

The results described here provide the first comparison of DNA constructs in mediating gene silencing in *Anopheles* and represent an important step towards the development of stable and heritable RNAi in these important malaria vectors.

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