Joining of long double-stranded RNA molecules through controlled overhangs

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ABSTRACT
We describe two methods for creating long (>1 kb) dsRNA molecules with specific, user-controlled overhangs for efficient hybridization and ligation. The two methods create double-stranded RNA (dsRNA) molecules with 5’ overhangs or with 3’ overhangs using T7 RNA polymerase (T7 RNAP) in transcription reactions of carefully designed PCR products. Primers utilized in the PCR reactions provide the template for the desired dsRNA overhangs. These methods provide complete control of the length and the sequence of the overhangs. This supplies a tool which is particularly lacking in dsRNA biochemistry given the absence of restriction endonucleases active on these substrates.

INTRODUCTION
The creation of specific overhangs (‘sticky ends’) for double-stranded DNA (dsDNA) by restriction endonuclease digestion and the resulting control of DNA ligation have revolutionized molecular biology over the past few decades, permitting such varied applications as the creation of specific plasmids and constructs with which to study protein–DNA interactions, the controlled modification of genes and many others. Similar control of double-stranded RNA (dsRNA) would greatly enhance the flexibility of working with this important molecule. This is especially important since the discovery of RNA interference has demonstrated that the role of long dsRNA molecules in the cellular life cycle is much more important than previously imagined (1,2).

One of the ingredients lacking in the dsRNA toolkit is sequence-specific dsRNA endonucleases (3) with which to create sticky ends on dsRNA molecules. Here, we demonstrate that this problem may be circumvented by protocols that produce dsRNA with user-controlled, specific overhangs. These protocols are flexible since they function on any DNA substrate and allow the user to determine the sequence of the overhangs incorporated. An important example of the utility of sticky ends for dsRNA is controlled, specific and efficient ligation of dsRNA molecules. This is not possible with blunt-ended dsRNA molecules due to the lack of hybridization. This makes it difficult to couple specific molecules to each other, results in a lack of control of the orientation of ligation partners with respect to each other, and decreases the ligation efficiency enormously due to the decreased time that the ligation partners spend in proximity. Effective creation of dsRNA with sticky ends will therefore facilitate studies of protein binding to dsRNA (4) by the creation of appropriate substrates, the implementation of DNA–RNA networks in biotechnology, and the inclusion of dsRNA in single molecule measurements (5–9).

In addition, it will facilitate RNA labelling and the creation of long dsRNA molecules with overhangs tailored to RNA–protein interactions (e.g. Dicer, which prefers a 2-nt 3’ overhang).

MATERIALS AND METHODS
Oligonucleotide design
All PCR primers were purchased from Isogen Life Science (Maarsen, The Netherlands). We indicate the sequences of primers used to build overhangs into dsRNA molecules, using F to designate a forward primer and R to designate a reverse primer. Within the primers, the T7 promoter site is underlined, brackets indicate the part of the primer that is not complementary to the template DNA, and the future overhangs of the molecules are shown in italics.

1. Use of the 3’-protocol to make 7.4 kb dsRNA with 4 nt overhangs. The DNA substrate used is Litmus 28i (New England Biolabs) with an insert from λ DNA. This substrate already contained two opposing T7 promoters separated by 7.4 kb, while the 3’-protocol in its most general form (Figure 1A) allows the user to incorporate the T7 promoters should they be absent on the substrate. The only consequence is that primers F1, R2 do not include the T7 promoters and anneal 3’ to the complements of the promoter sequences on the substrate.

Main molecule, 7.4 kb long:
F1  CTATGACCATATATTACGCAAGC
R1  [AGCT]GGCCCTTAAGCACGGGTACC
F2  [GTAC]GGCAGATCCACTGATTTCT
R2  CAAAGGCATTAGTGGTAAACG

The presence of overhangs was tested by ligation of the 7.4 kb dsRNA to 0.4 kb dsDNA fragments.

2. Use of the 5’-protocol to make 4.2 kb dsRNA with 4 nt overhangs. The DNA substrate used is recombinated pBAD vector.

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Figure 1. Protocols for making long dsRNA with sticky ends (DNA sequences, uppercase; RNA sequences, lowercase). The sequence of the T7 promoter site is 5'-TAATACGACTCACTATAGG-3'. (A) Protocol for making long dsRNA with 3' overhangs. Starting from a DNA template, two primer pairs are selected: each primer pair is used to build a T7 promoter site into one side of the PCR product and an overhang sequence (italicized) into the other side of the PCR product. The PCR products are subsequently combined in a transcription reaction using T7 RNAP. This results in the formation of a dsRNA molecule with distinct 3' overhangs. (B) Protocol for making long dsRNA with 5' overhangs. As before, two primer pairs are selected: each primer pair is used to incorporate a T7 promoter site directly followed by an overhang sequence (italicized) into a PCR product. The PCR products are subsequently combined in a transcription reaction with T7 RNAP. This results in the formation of a dsRNA molecule with two distinct 5' overhangs.
Main molecule, 4.2 kb long:
F1 [TAATACGACTCACTATAGGG]AAGATTTAGCC-GATCCTACCTGAC
R1 GGTTAACCCTCAACTCCATTTC
F2 AAGATTAGGGATCTACCTGAC
R2 [TAATACGACTCTATAGGGG]AAGTTAACCCTCAACTCATTTCC

The presence of overhangs was tested by ligation of the 4.2 kb dsRNA to 0.4 kb dsDNA fragments.

3. Use of the 5'-protocol to make 0.4 and 4.2 kb dsRNA with 8 nt overhangs. The DNA substrate used is recombinated pBd vector for the 4.2 kb dsRNA and bacteriophage λ DNA for the 0.4 kb fragments.

Main molecule, 4.2 kb long:
F1 [TAATACGACTCTATAGGGC]ACGATTTAGGGATCCTACCTGAC
R1 GGTTAACCCTCAACTCCATTTC
F2 AAGATTAGGGATCTACCTGAC
R2 [TAATACGACTCTATAGGGG]AAGTTAACCCTCAACTCATTTCC

Digoxigenin-labelled ends, 0.4 kb long:
F1 [TAATACGACTCTATAGGGC]ACGATTTAGGGATCCTACCTGAC
R1 GGTTAACCCTCAACTCCATTTC
F2 AAGATTAGGGATCTACCTGAC
R2 [TAATACGACTCTATAGGGG]AAGTTAACCCTCAACTCATTTCC

Biotin-labelled ends, 0.4 kb long:
F1 [TAATACGACTCTATAGGGC]ACGATTTAGGGATCCTACCTGAC
R1 GGTTAACCCTCAACTCCATTTC
F2 AAGATTAGGGATCTACCTGAC
R2 [TAATACGACTCTATAGGGG]AAGTTAACCCTCAACTCATTTCC

Transcription reaction
Each 60 µl transcription reaction (HiScribe RNAi transcription kit, New England Biolabs) contained 0.5–1 µg of each PCR template, 300 U T7 RNAP, High Molecular Weight (HMW) Component Mix (0.67 mM Tris–HCl, pH 8.1, 0.05 mg/ml BSA, 3.33 units/ml inorganic pyrophosphatase, 400 units/ml pancreatic ribonuclease inhibitor and 1.67% glycerol), transcription buffer (40 mM Tris–HCl, pH 8.1, 19 mM MgCl₂, 5 mM DTT, 1 mM spermidine), and NTPs (4 mM each). The samples were incubated for 3 h at 37°C.

Labelling dsRNA with biotin and digoxigenin
Incorporation of biotin-16-UTP and digoxigenin-11-UTP nucleotides into 0.4 kb dsRNA was executed by the addition of 1 molar unit modified UTP for every 2 molar units UTP in the transcription reactions.

Hybridization protocol
To improve the efficiency of strand annealing of the transcription products, the following step directly succeeded the 3 h transcription reaction: the reactions were heated to 65°C for at least 1 h in the presence of 30 mM EDTA and cooled down to room temperature by slowly decreasing the temperature (1.25°C/5 min) in a Mastercycler with heated lid (Eppendorf). Yields of up to 500 ng of 8 kb dsRNA and 2.5 µg of 0.4 kb dsRNA were obtained per 30 µl transcription reaction.

Purification
Following hybridization, the dsRNA transcription reactions were purified using an RNasey MinElute Cleanup Kit (Qiagen). They were then treated with 5 U of RNase-free DNase I (Roche Applied Science) for 1 h at 37°C and purified.

(De)phosphorylation
5'-triphosphates were replaced by 5'-monophosphates using a KinaseMax 5' End-Labeling Kit (Ambion, Huntingdon, UK). Each 17 µl dephosphorylation reaction, containing 0.1 U calf intestine alkaline phosphatase (CIP), dephosphorylation buffer, and 0.1 (8 kb) to 10 (0.4 kb) pmol dsRNA was incubated for 1 h at 37°C. The reactions were purified by addition of 17 µl phosphatase removal reagent (P RR) and retrieval of the supernatant. Phosphorylation of the 5' end was carried out in 35 µl reactions containing 0.57 mM ATP, kinase buffer, and 15 U polynucleotide kinase (PNK) and incubated for 1 h at 37°C. The reactions were purified using Qiagen RNasey spin columns.

DNA ligation partners
Labelled 0.4 kb dsDNA fragments for ligation to 7.4 kb dsRNA with 3' overhangs (Figure 1A) were synthesized in two PCR reactions using a pBADb10 substrate. One primer in each PCR reaction included a restriction site (for SacI or KpnI, respectively) 3 nt from the 5' end. Biotin-16-dUTP and digoxigenin-11-dUTP nucleotides were incorporated during the PCR reaction by the addition of 1 molar unit modified dUTP for every 2 molar units dTTP. The PCR products were then digested using SacI and KpnI, respectively. Labelled 0.4 kb dsDNA fragments for ligation to 4.2 kb
dsRNA with 5′ overhangs were made similarly except that the
primers included the restriction site for BsmA1 as well as
adjoining sequences to yield the correct overhangs upon diges-
tion. The efficiency of restriction endonuclease digestion may
decrease due to the presence of labelled nucleotides, but is
typically not found to decrease below 50%.

Ligation
The dsRNA–dsDNA ligation reactions (total volume 0–100 µl)
consisted of up to 100–500 ng 4.2 kb dsRNA, a 4-fold molar
excess of both 0.4 kb dsDNAs, Quick Ligase buffer and 2 µl T4 DNA Quick Ligase (New England Biolabs),
20 U SacI and 20 U KpnI if necessary to prevent dsDNA–
dsDNA ligation, and 10 µg BSA. These ligations were run at
16°C overnight and purified using Qiagen RNeasy spin col-
umns. The dsRNA–dsRNA ligation reactions (total volume
0–100 µl) using T4 DNA ligase consisted of up to 100–500 ng
4.2 dsRNA, a 5-fold molar excess of both 0.4 kb dsRNAs,
Quick Ligase buffer and 2 µl T4 DNA Quick Ligase (New
England Biolabs), and 40 U of Protector RNase Inhibitor
(Roche Applied Science, Germany). These ligations were
incubated at 16°C overnight and purified using Qiagen
RNeasy spin columns. The dsRNA–dsRNA ligation reactions
(total volume 10 µl) using T4 RNA ligase 2 consisted of up to
100–500 ng 4.2 dsRNA, a 5-fold molar excess of both 0.4 kb
dsRNAs, buffer (50 mM Tris-acetate pH 6.5, 40 mM NaCl,
5 mM DTT, 1 mM MgCl₂), 1 mM ATP, 1 µl T4 RNA ligase 2
and 20 U of Protector RNase Inhibitor (Roche Applied
Science, Germany).

Gel electrophoresis
All gel electrophoresis analyses shown in the figures were
conducted on a 0.75% agarose gel with an electric field of
4 V/cm.

RESULTS
DsRNA molecules with 3′ overhangs can be made using the
following protocol (Figure 1A). Two PCR products are gen-
erated in separate reactions PCR 1 and PCR 2, both containing
a single promoter sequence for T7 RNA polymerase (T7
RNAP). Each PCR product hence forms a template for the
transcription of a single strand of RNA. In PCR 1, the forward
primer (F1) incorporates the T7 promoter sequence, while the
reverse primer (R1) consists of (from 5′ to 3′) an overhang
sequence (AGCT in Figure 1A), two guanine residues and a
sequence complementary to the DNA substrate. In PCR 2,
the forward primer (F2) consists of (from 5′ to 3′) an overhang
sequence (GTAC in Figure 1A), two guanine residues and a
sequence complementary to the DNA substrate; the reverse
primer (R2) is used to build in the T7 promoter sequence.
In our example, the two overhang sequences in PCR 1 and PCR 2
contain an identical number of nucleotides, but this need not
generally apply. In a single reaction, both PCR products are
then transcribed into RNA by T7 RNAP, starting with the two
guanines at the 5′ end of the promoter sequence which are a
prerequisite for efficient transcription (11). This yields tran-
scripts which are entirely complementary save at the 3′ ends.
Subsequent hybridization of these strands yields a dsRNA
molecule with two distinct 3′ overhangs. Note that the two
guanine residues incorporated into primers F2, R1 are
transcribed into cytosines which hybridize with the two
5′-guanines synthesized by T7 RNAP on the opposite strand
(Figure 1A).

DsRNA molecules with 5′ overhangs can be made by choosing
primer configurations such that the promoter site and the
overhang sequence are part of a single primer (Figure 1B). In
PCR 1, the forward primer (F1) consists of (from 5′ to 3′) the
T7 promoter sequence, an overhang sequence (ATCGGCC in
Figure 1B), and a sequence complementary to the DNA sub-
strate. The reverse primer (R1) anneals entirely to the DNA
substrate. In PCR 2, the forward primer (F2) anneals entirely to
the DNA substrate, while the reverse primer (R2) consists of
(from 5′ to 3′) the T7 promoter sequence, an overhang
sequence (GCTACC in Figure 1B), and a sequence comple-
mentary to the DNA substrate. The single-stranded RNA
transcripts of the two PCR products are complementary except
at the 5′ ends, where they contain (from 5′ to 3′) two guanines
followed by the overhang sequences. Subsequent
hybridization of these strands yields a dsRNA molecule with
two distinct 5′ overhangs, 5′-GGAUCGCC-3′ and
5′-GGGGCAUCC-3′.

In both protocols, the specific choice of overhangs is deter-
mined by the user. Thus, the protocols permit the creation of
sticky ends that are palindromic (so that they can be ligated to
dsRNA fragments digested by standard restriction endonu-
clases) as well as the creation of sticky ends that are not
palindromic. The latter can present an advantage as it prevents
unwanted creation of dimers. Of course, one can also consider
combining the two protocols to create dsRNA molecules with
a 5′ overhang on one side and a 3′ overhang on the other side.

We now demonstrate the synthesis and ligation of dsRNA
molecules using these protocols. We synthesize three long
dsRNA molecules using these protocols and demonstrate
the successful incorporation of sticky ends by ligating them
to both dsDNA and dsRNA fragments. For an application
in single-molecule biophysics, we seek to create a long
(4 kb or larger) main dsRNA molecule ligated at each end
to short 0.4 kb fragments that are multiply labelled with biotin
and digoxigenin, respectively. This will therefore constitute
our example reaction, of which the potential products are the
main molecule plus a biotin fragment, the main molecule plus
digoxigenin fragment and the main molecule plus both
labelled fragments. The latter three-body reaction is less effi-
cient but can be observed in ligation reactions with T4 RNA
ligase 2 [Rnl2, gift of Stewart Shuman (12)] and by using
single-molecule techniques, as we explain below.

First, we demonstrate the creation of dsRNA molecules with
3′ overhangs and their ligation to dsDNA fragments. The
primers were chosen as described above to yield 4 nt over-
hangs 5′-GUAC-3′ and 5′-AGCU-3′ (Figure 1A), which are
complementary to DNA overhangs created by the restriction
endonucleases KpnI and SacI. Following formation of the
7.4 kb PCR products, a transcription reaction was run. Gel
electrophoresis following this reaction (Figure 2A) showed
two bands: the dsDNA PCR products (denoted DNA) and a
second, bright band which migrated more slowly than the
dsDNA PCR products (denoted RNA). Several controls indi-
cated that this retarded band consisted of dsRNA: (i) transcrip-
tion reactions run using only a single PCR product did not
display this band; (ii) addition of RNase-free DNase I to the
transcription reaction product removed the band corresponding to the dsDNA PCR products, but not the retarded band (Figure 2B); (iii) addition of a mixture of RNases (which digests ssRNA) to the transcription reaction product did not remove the retarded band; (iv) addition of RNase III (which digests dsRNA) to the transcription reaction product did remove the retarded band. Therefore, we concluded that this retarded band corresponded to dsRNA. Next, using T4 DNA ligase, we ligated this 7.4 kb dsRNA molecule to 0.4 kb labelled dsDNA fragments that had been digested with KpnI and SacI. In order to limit dsDNA–dsDNA ligation (probably since the efficiency of dsDNA–dsRNA ligation greatly exceeds the efficiency of dsDNA–dsDNA ligation when using T4 DNA ligase [Paul Walsh, NEB, personal communication; (13)], we included the restriction endonucleases KpnI and SacI in the reaction. This greatly improved the final yield of the dsDNA–dsRNA construct (Figure 2C, band denoted RNA + DNA ends), presumably since the restriction endonucleases cleave ligated dsDNA dimers but do not cleave dsDNA fragments ligated to dsRNA. This joining of dsDNA fragments to dsRNA molecules demonstrates the successful incorporation of sticky ends with the correct sequences into dsRNA.

Next, we synthesize dsRNA molecules with overhangs using the 5′-protocol (Figure 1B). Using two 4.2 kb PCR templates which encoded for overhangs 5′-GGTT-3′ and 5′-GGGAA-3′, a transcription reaction was run as previously, yielding a more slowly moving band (denoted dsRNA) above the PCR products (Figure 3A). As before, the transcription product was then digested with RNase-free DNase I which removed the faster band. This confirmed that the retarded band in the transcription reaction was dsRNA (Figure 3B). T7 RNAP synthesizes a ribonucleotide polymer with one NTP at the 5′ end. For optimal ligation, this NTP can be converted into an NMP. The 4.2 kb dsRNA fragment was therefore treated with CIP and T4 polynucleotide kinase to obtain monophosphates at its 5′-termini prior to ligation (this was not found to be efficient in the 3′-protocol, see below). The ligation partners for the 4.2 kb dsRNA molecules were two labelled dsDNA molecules which had been digested with BsmA1 to yield overhangs 5′-CCAA-3′ and 5′-CCTT-3′, respectively. These overhangs are complementary to those incorporated into the 4.2 kb dsRNA molecule. Given these non-palindromic overhangs, the dsDNA molecules could not ligate to each other, but they could ligate to the RNA overhangs. Following ligation (Figure 3C), an additional band appeared in the gel (denoted RNA + DNA ends) which migrated more slowly than the 4.2 kb dsRNA fragment, as expected. This demonstrated that the 5′-protocol could also be used to successfully incorporate overhangs into dsRNA molecules.

Finally, we demonstrate that dsRNA molecules can be joined to other dsRNA molecules using the 5′-protocol (Figure 1B). For this, we construct three dsRNA molecules with 5′ overhangs (one 4.2 kb molecule with 8 nt overhangs 5′-GGGCGUACC-3′ and 5′-GGGAUCGCC-3′ and two 0.4 kb labelled fragments with overhangs 5′-GGUAGCCC-3′ and 5′-GGGCUACC-3′, respectively). The transcription reaction and the control with DNase I are shown for the 4.2 kb dsRNA (Figure 4A). All three dsRNA molecules were subsequently treated with CIP and kinase. In the case of dsRNA–dsRNA ligation, this step is essential since its omission results in the complete absence of 5′-monophosphates in all ligation partners, and hence in the absence of any ligation product. Following this treatment, the ligation reaction was carried out, resulting in additional bands corresponding to ligation to one or both labelled fragments (Figure 4B, denoted RNA + RNA ends). This demonstrated that the 5′-protocol can be used to
join to dsRNA molecules to each other. However, since the longer length of the overhangs incorporated into these molecules (8 nt compared to 4 nt utilized in the previous ligations) implied that hybridization products could be stably formed at room temperature, we applied an additional control to distinguish hybridization from ligation. An identical reaction to the one described in Figure 4B was carried out, but in the absence of T4 DNA ligase. Gel electrophoresis of the reaction product at room temperature showed a pattern similar to Figure 4B, which indicates that there is good hybridization between the molecules. Next, the products of both reactions were heated to 55°C (a temperature well above the melting temperature $T_m$ of the overhangs) for 15 min, rapidly chilled on ice and run on gel again. In the reaction without ligase (Figure 4C, left lane), only the band corresponding to the 4.2 kb dsRNA molecule remained, whereas in the reaction with ligase (Figure 4C, right lane), both the 4.2 kb dsRNA band and a longer band remained. The fact that a longer band remained in the presence of heating demonstrated ligation of the phosphodiester backbone. A similar experiment using Rnl2, which is a ligase specific to dsRNA, confirmed this result and demonstrated an increased ligation efficiency (Figure 4D).

For all three reactions described above, the products were successfully tethered in a single-molecule apparatus and measured to have correct lengths (J. A. Abels, F. Moreno-Herrero, T. van der Heijden, C. Dekker, and N. H. Dekker, submitted). Such tethering of molecules is only possible in the presence of biotin- and digoxigenin-labelled extremities. Furthermore, both gel electrophoresis and single-molecule analysis of ligation reactions with blunt-ended dsRNA molecules yielded null results (data not shown). This demonstrates the utility of the RNA sticky ends generated using the above protocols.

**DISCUSSION**

We now compare the two protocols to create dsRNA molecules with sticky ends. While both provide the user with great flexibility by allowing the creation of dsRNA molecules with sticky overhangs from any DNA substrate, the 3’-protocol is the more versatile of the two techniques because the entire sequence of the overhang can be set by the user (Figure 1A). This is not strictly true for the 5’-protocol if high efficiency transcription is required, because T7 RNAP is most efficient when the last two nucleotides of the promoter sequence are guanines (11). The two corresponding 5’-ribonucleotides will form part of the 5’ overhang sequence; nevertheless, the remainder of the overhang sequence can be freely chosen (Figure 1B). The choice of protocol may also depend on whether one aims to join dsRNA molecules through hybridization or through ligation. Since dephosphorylation/phosphorylation (an important consideration with dsRNA–dsRNA ligation) is significantly more efficient on 5’ overhangs than on 3’ overhangs (14), ligation may proceed more efficiently using the 5’-protocol. Alternatively, the 3’-protocol can be adapted by including strategies for increasing the efficiency of dephosphorylation and phosphorylation of 3’ overhangs [such as temporary heating and rapid chilling (14)] or by allowing the two single-stranded RNA molecules generated during transcription to hybridize only following incorporation of monophosphates.

Next, we discuss the fidelity of T7 RNAP. In certain cases, T7 RNAP does not faithfully generate the 5’ end or the 3’ end of its transcript (10,15,16). Such errors would generate sticky ends which deviate from the intended overhang sequences, which, though it need not prevent successful joining of dsRNA molecules by hybridization of long sticky ends,
might prohibit ligation of dsRNA molecules. However, the fidelity of T7 RNAP can be maximized in several ways: by preventing cases where the first five nucleotides transcribed by T7 RNAP are all guanines (thought to cause errors in the 5′ end of the transcript due to slippage of the RNA polymerase) and by utilizing the technique developed by Kao et al. (10) in which the PCR primers include several 5′ end methoxy nucleotides (which prevents errors in the 3′ end of the transcript due to the addition of nontemplated nucleotides).

Finally, we discuss the efficiency with which these protocols yield dsRNA with the correct overhangs. This efficiency can be estimated from the ligation reactions (it is necessarily a lower bound since the ligation efficiency may not attain 100%). The experiments with Rnl2 demonstrate a ligation efficiency ≳ 50% (Figure 4D, right lane) using the 5′-protocol, indicating very successful synthesis of dsRNA with the correct overhangs. We have further investigated whether the presence of labeled nucleotides in or near the overhangs (Figure 1B) affects this figure. For the labelling conditions described in the Materials and Methods, however, we have determined that this is not the case.

In conclusion, the protocols which we have demonstrated provide a much needed tool lacking in the molecular biology of dsRNA with which to prepare dsRNA molecules with sticky ends. We expect that this can be used in various settings such as the creation of dsRNA molecules for specific dsRNA–protein interactions and the efficient labelling of dsRNA molecules, and that, in general, it greatly enhances the flexibility of working with this molecule.

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