A yeast RNA-hybrid system for the detection of RNA–RNA interactions in vivo

NICOLAS PIGANEAU, URSULA E. SCHAUER, and RENÉE SCHROEDER
Max F. Perutz Laboratories, Department of Biochemistry, University of Vienna, A-1030 Vienna, Austria

ABSTRACT

RNA–RNA interactions play a crucial role at many different levels of the cellular metabolism such as plasmid replication control, viral encapsidation, or transcriptional and translational regulation. Therefore, methods are necessary to investigate the molecular determinants of given interactions, including their stabilities, or to screen for new interacting partners. We designed an RNA-hybrid system in *S. cerevisiae*, based on the yeast three-hybrid system. In this setup, the activation of a reporter gene is dependent on the interaction of two RNAs. A loop–loop interaction similar to the dimerization initiation site of the HIV genome was used as a model system, demonstrating that in this novel RNA-hybrid system only cognate RNAs promote the activation of the reporter gene. Levels of reporter activation correlate well with interaction stabilities determined in vitro by UV melting analyses, suggesting that conditions used for the analysis of in vitro structural stabilities translate well into the intracellular environment. Furthermore, the system was applicable for a screen against a test library. Nine out of ten selected clones were identified as predicted interaction partners for the bait RNA. In summary, we present a yeast reporter system depending on RNA–RNA interactions, which can be used alternatively for analysis of known interactions or for screening libraries in search for new interaction partners.

Keywords: in vivo RNA–RNA interaction; loop–loop interaction; yeast three-hybrid system; intracellular RNA structural stability

INTRODUCTION

RNA–RNA interactions in cellular metabolism are gaining in prominence with the discovery that a large number of transcripts in higher eukaryotes are noncoding RNAs (ncRNAs). For example, in mouse cDNA libraries, almost half of the transcripts are ncRNAs (Mattick 2003). It is therefore anticipated that the functional RNAs already identified constitute only the tip of the iceberg. Frequently, the interaction partners of ncRNAs are not only proteins, but also other RNA molecules. Classic examples of interactions involving two RNA molecules are snRNAs (Forne et al. 1996); snoRNAs with their targets (Bachellerie et al. 2002); micro-RNAs from the RNAi pathway with their target mRNA(s) (Ambros 2004; Murchison and Hannon 2004); ncRNAs from *Escherichia coli*, also called sRNAs (Hershberg et al. 2003; Repoila et al. 2003); and loop–loop interactions (Brunel et al. 2002).

There is a need for efficient methods allowing the detection of RNA–RNA interactions and the screening of genomes for new targets of RNAs. In the past, the yeast two- and three-hybrid systems (Fields and Song 1989; SenGupta et al. 1996) proved essential in the examination of the networks of protein–protein and RNA–protein interactions. However, the building blocks necessary for the design of a similar system applicable to RNA–RNA interactions were not available until RNA by itself was demonstrated to be able to promote transcription (Sengupta et al. 1999; Buskirk et al. 2003; Saha et al. 2003). We reasoned that, as for their protein counterparts, it should be possible to separate the signal responsible for promoter localization (in this case, a MS2-RNA binding domain to a lexA-MS2 coat-protein fusion) from the activation domain.

Using this strategy, we designed a new system termed the “RNA-hybrid system.” With this new method, it should be possible to investigate the determinants of known RNA–RNA interactions in vivo. Furthermore, this system would also be suited for library screenings and the identification of unknown interaction partners of RNAs. To demonstrate the feasibility of this system, we applied it to the HIV-1 type dimerization signal loop–loop interactions.

Intermolecular loop–loop interactions, also called kissing complexes, are widely spread and serve a diverse range of

Reprint requests to: Renée Schroeder, Max F. Perutz Laboratories, Department of Biochemistry, University of Vienna, Dr. Bohrgasse 9/5, A-1030 Vienna, Austria; e-mail: renee.schroeder@univie.ac.at; fax: +43-1-4277-9522.

Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.2105506.
biological functions. Loop–loop interactions are implicated in plasmid replication control and involve, for example, the RNAs CopA–CopT or RNA I–RNA II (Eguchi and Tomizawa 1990). Another well-known instance of kissing interaction is the dimerization initiation signal (DIS) loop responsible for initiation of the dimerization of the genomic HIV RNA (Paillart et al. 1996). This kissing complex between two DIS-loops is the initial transitory structure, precluding a refolding of the two molecules leading to the formation of an extended duplex between the two copies of the HIV genome (Laughrea and Jette 1996; Brunel et al. 2002; Windbichler et al. 2003). Our laboratory previously determined the thermodynamic parameters governing the DIS kissing complex (Weixlbaumer et al. 2004).

In this article, we show that the RNA-hybrid system can efficiently distinguish between cognate and noncognate RNAs from two different kissing complexes differing only in the interacting loops. The β-galactosidase activity obtained with the RNA-hybrid system correlates with the stability of the interaction as determined by physicochemical methods in vitro. This demonstrates that the nonphysiological conditions (1 M NaCl) used to quantify the stability of RNA structures in vitro translate well to the intracellular conditions. Furthermore, we confirm the potential of the RNA-hybrid system for the screening of new interaction partners of known RNAs.

RESULTS

Design of plasmids and transcripts

The RNA-based transcriptional activator (m26-11) was evolved by Buskirk et al. (2003) from a random library cloned in plIIa MS2-2. This RNA molecule activates transcription in a yet unknown mechanism when tethered to the promoter of a reporter gene through the binding of the MS2-RNA to the LEXA-MS2 coat-protein fusion. In order to monitor RNA–RNA interactions, the simplest design imaginable was to separate the RNA transcriptional activator from the DNA tethering sequence (MS2 binding domain) and fuse both domains to interacting RNAs, allowing the formation of an activation complex only via the interaction of the two hybrid RNA molecules.

The basic strategy for the analysis of RNA–RNA interactions using the RNA-hybrid system is shown in Figure 1A. Yeast strain YBZ-1, from the three-hybrid system (Bernstein et al. 2002; Hook et al. 2005), carries both lacZ and HIS3 reporter genes under the control of multiple lexA operators. When two interacting hybrid RNAs are coexpressed, the MS2–RNA X hybrid binds to the MS2 coat-protein fusion and tethers the RNA Y–m26 fusion RNA to the promoter of lacZ and HIS3, thereby activating gene expression. (B) Overview of plasmids pAN MS2-2 and plIIa-m26 used in the RNA hybrid system. RNA X and RNA Y can be cloned into pAN MS2-2 (respectively, plIIa-m26) between the restriction sites XmaI and SphI (SphI and Sall). The schematic structure of the hybrid RNA is shown next to the corresponding plasmid with the positions of the respective restriction sites indicated. (C) Sequence and secondary structure of two pairs of RNAs used in this study.

Our principal concerns during the design of the RNA-hybrid vectors were RNA localization and folding. To promote colocalization of both RNAs in the nucleus, they are expressed from the same RNase P RNA (RPR) promoter used in the classical three-hybrid system. This ensures that both RNAs have identical promoter, leader, and 3′ tail sequences. Thus, we constructed plasmid plIIa-m26 and plasmid pAN MS2-2 (Fig. 1B). Plasmid plIIa-m26 is a derivative of m26-11 in which the MS2-RNA binding sequence was deleted via simple excision of the Sall fragments containing the MS2-RNA tand repeats. To construct pAN MS2-2, we cloned the RNA expression cassette from plIIa MS2-2 in the shuttle vector pACTII. When inserting potential interaction partners in both plasmids, special care was taken to preserve
the secondary structure of the m26 activation domain, as described below.

**Loop–loop interactions**

We used the HIV-1 type DIS-loop as a model for the analysis of RNA–RNA interactions using the RNA-hybrid system. The DIS-loop is a hairpin structure that contains a 6-nt self-complementary loop sequence flanked by a double and a single adenine (Paillart et al. 1996). Self-complementarity would be detrimental in this case, as it would lead to the formation of homodimers (X–X or Y–Y) instead of heterodimers (X–Y). We therefore use nonpalindromic loop sequences throughout this study. First, two different stem-loop pairs were cloned into pAN MS2-2 and pIIIa-m26. To guarantee that the transcribed RNAs fold correctly, we analyzed the secondary structure of different constructs using the mFOLD algorithm (Zuker 2003). This is of particular importance in the case of the activator domain m26, which is located at a three-way junction.

X₁ and X₂ stem-loops were fused to the MS2-RNA domain in pAN MS2-2. The loop of each X RNA contains 6 nt cognate to the respective Y RNA loop, preceded by two adenines and followed by one adenine (Fig. 1C). The related Y₁ and Y₂ stem-loops were cloned in pIIIa-m26. To facilitate independent folding of the m26 domain and of the Y RNAs, we lengthened the stem originally comprising the MS2-RNA tandem repeats (Fig. 1C). The stems containing X₁ and X₂ were chosen as to ensure correct folding. In the case of X₂, the stem is 1 G–C bp longer than the X₁ stem. We first tested the expression of both RNAs in yeast by Northern blot analysis with a probe hybridizing to both RNAs (Fig. 2A). As expected, two bands of equal intensity are observed, indicating that both RNAs are expressed at the same level.

We then tested the reporter system by using a quantitative β-galactosidase assay (Fig. 2B). As can be seen, β-galactosidase activity is dependent on the coexpression of two cognate RNAs. When X₁ (respectively, X₂) is expressed in the presence of Y₁ (Y₂), the reporter gene activity is increased 10-fold over the background, while coexpression of Y₁ (Y₂) fails to activate lacZ expression. RNAs Y₁ and Y₂ only differ in the loop sequence (AAGGUCGGA and AAGGCACGA, respectively). Hence, the difference observed between lacZ expression when coexpressed with X₁ and X₂ can be attributed to the loop–loop interaction. In comparison to the three-hybrid system, the expression levels are low (eightfold reduction). Intermolecular reporter activation (X₁–Y₁) is 36-fold weaker than intramolecular activation (m26-11). This reduction is comparable with the 22-fold reduction in activity observed between the full-length GAL4 protein and the two fusion proteins interacting in the two-hybrid system (Fields and Song 1989).

We ensured that the difference observed between cognate and noncognate RNAs with the β-galactosidase assay was reproducible by using the HIS3 reporter gene. Whereas all
transformed yeast strains can grow on media lacking leucine and uracil, only cells expressing cognate RNAs are able to grow on medium also lacking histidine, indicating that HIS3 expression is turned on in these cells only (Fig. 2C). When observing growth under more stringent conditions using 1 mM 3-aminotriazole (3-AT), we see a higher HIS3 expression of the three-hybrid system positive control compared with X1–Y1, confirming the results obtained with the β-galactosidase assay.

We have also measured the β-galactosidase activity of clones with a longer loop sequence. In X50 and Y50 (Fig. 4A), the stem sequence is 2 bp longer, and a 50-nt sequence from the gene StpA (Zhang et al. 1995) is introduced between the flanking adenines. The arbitrarily chosen StpA coding sequence was searched for a 50-nt stretch with the lowest possible secondary structure stability. The pair was tested against its complement and against the empty RNAs transcribed from pAN MS2-2 and pIIIa-m26. As can be observed in Figure 2B, β-gal expression is activated at levels comparable to the pairs X1–Y1 and X2–Y2 when both cognate RNAs are expressed (Fig. 2B). We were surprised that the levels of activation resulting from an interaction over 50 bp is not higher than the signal obtained with a paring over six bases. The Northern blot (Fig. 2A) reveals that the RNA containing X50 seems to be expressed at a lower level compared with X1 and X2. Other explanations for this result could be the longer distance between the promoter and the activation domain, or simply that the levels of reporter activation observed for the different pairs are already maximal.

Signal intensity reflects the stability of interaction

Our laboratory previously investigated the stability of kissing loop interactions via UV melting analysis (Weixlbaumer et al. 2004). These constructs (including the pair X1–Y1) were cloned and tested in the RNA-hybrid system (Fig. 3). The only difference between the X1–Y1 pair and the pairs 1a to 1c is the sequence of the interacting loop region that was replaced in X1a to X1c and accordingly in Y1a to Y1c. We could observe a direct correlation between the β-galactosidase activity and the interaction stability measured via UV melting analysis. The most unstable pair used (X1c–Y1c), interacting with two G–C base pairs and four A–U base pairs, displays a melting temperature of 32°C and a free energy at 37°C of only −7.5 kcal/mol. This interaction can barely be detected using the RNA-hybrid system (Fig. 3).

It should be noted that the theoretical stability of the pair X2–Y2 (−12.8 kcal/mol) should lead to an activity between that of the pairs X1–Y1 and X1b–Y1b (cf. Figs. 2B and 3). However, the stem of X2 contains an additional base pair and cannot be directly compared with the other constructs. We suggest that this additional base pair changes the orientation of the activation domain on the lacZ promoter and is the cause of the lower activity observed (Fig. 2B).

Influence of base-pairing length

One major application of the RNA-hybrid system would be the identification of new interaction partners through library screening. However, if 6 bp are sufficient to produce full β-galactosidase activity, many false positives would be detected during a screening for new interaction partners. To investigate this problem, we designed derivatives of X50 and Y50 in which the base-pairing between both constructs was reduced to 40, 30, 20, 10, 8, and 6 nt in the center of the loop sequence (for sequences, see Fig. 4A) while the length of the loop remains constant. In the center of these constructs, six bases were exchanged to CCGACC in the X constructs and to the complementary sequence in the Y construct in order to introduce the same base-pairing as between X1 and Y1 and hence the same duplex stability. To avoid base-pairing outside of the desired region in the constructs X50mod-6 to X50mod-40, the nucleotides outside of the interacting region were substituted with the corresponding sequence of the Y construct (i.e., the sequence of the loop of X50-6 is the sequence of the Y50 loop outside of the central six bases). Unexpectedly, the modified constructs interacting over 50 bp (X50mod and Y50mod) display a lower β-galactosidase activity than do the unmodified constructs (Fig. 4). One possible explanation for this is that the loops of both X50mod and Y50mod can form internal secondary structures more stable than the loops of X50 and Y50 (−2.9 kcal/mol and −5.4 kcal/mol).
molecules; in the case of the HIV genome, however, it is sufficient for initiation of dimerization. Furthermore, the interaction between two RNAs in the context of the DIS-loop is more stable than the corresponding RNA duplex (Weixlbaumer et al. 2004).

Screening a library for interaction partners

Since interacting RNAs demonstrate a growth advantage on medium lacking histidine in comparison to noncognate RNAs, the screening of a test library was performed. A library containing a 6-nt random region in place of the X2 loop was constructed and inserted into pAN MS2-2. The number of yeast transformants after introduction of the library in YBZ-1 carrying plasmid pIIIa Y2 m26 was evaluated as 42,000 clones. Each of the 4096 possible sequences should therefore be present in ~10 copies on average. The transformants were plated on synthetic medium

**FIGURE 4.** Influence of base-pairing length on reporter activation. (A) Sequences of the different RNAs tested. Variants of X50 and Y50 were designed to interact over different lengths with a central sequence corresponding to the six interacting base pairs of X1 and Y1. The sequence shown encompasses the cloning sites and part of the stem enclosing the interacting loops. (B) Quantitative analysis of ß-galactosidase activity of the different pairs. The activity is normalized relative to the activity of X1–Y1 (1.0). Error bars indicate standard deviation. X50–Y50 indicates YBZ-1 strain transformed with plasmids expressing RNAs X50 and Y50; numbers 6–50, YBZ-1 strain transformed with plasmids expressing RNAs X50 mod-6 to X50 mod and Y50 mod.
lacking leucine and histidine with 0.2 mM 3-AT. Of 22 growing colonies, 10 were confirmed as potential interaction partners after streaking on the same medium. Sequences of the 10 clones are shown in Figure 5A. Eight clones carry the expected loop sequence from X2; the number of clones found fits nicely with the 10 expected copies of each sequence. One sequence (LibN1) displays a single point mutation C to U at the fifth position in the loop. This mutation transforms a G–C base pair in the original kissing loop complex into a G–U base pair. The sequence from clone LibN8 carries no visible similarity with the X2 loop sequence and cannot hybridize over >2 bp with Y2. The β-galactosidase activity of the different clones in the presence of Y2 and Y2 was measured (Fig. 5B). As expected, none of the clones activates lacZ expression when coexpressed with Y1. When tested against Y2, clone LibN8 revealed itself as a false positive, and clone LibN1 displayed a reduced expression compared with wild-type X2 and LibN2. This result is in agreement with the fact that β-galactosidase activity correlates with the stability of interaction as this mutation transforms a G–C base pair into a G–U base pair.

In conclusion, in a test library screening, 90% of the selected clones were identified as interaction partners for the bait RNA. This demonstrates the accuracy of our system and the dependence for the correct interaction for signal induction.

DISCUSSION

We present in this study a new system for the investigation of RNA–RNA interactions in vivo. Using a loop–loop interaction as a model system, we show first the ability of the system to distinguish between cognate and noncognate interaction partners, demonstrating that RNA–RNA interactions can be investigated with this method. Second, we show that the interaction stability is directly correlated with the signal strength. The RNA-hybrid system can therefore be used to compare different ligands quantitatively. Third, we used the method to screen a model library and retrieved interaction partners for the bait used, indicating that this system is also suitable to search for interaction partners of RNAs in library screens.

The level of reporter activation is low compared with the three-hybrid system, which could be detrimental when screening a large library due to the low signal-background ratio. It would therefore be of interest to increase the potency of the activation domain. In this regard, an investigation of its mode of action and exact molecular requirements would be helpful. Furthermore, we plan to introduce multimers of the activation domain into a given RNA molecule to increase activity.

Expression levels of the reporter genes in cis by m26-11 are very high, and it is conceivable that further evolution of this clone was hindered by the lack of sufficient stringency (Buskirk et al. 2003). Obviously in the trans situation, we are not facing the same problem. We therefore plan to try to evolve more potent activators in the RNA-hybrid system via random mutagenesis of the m26 domain and selection on high concentrations of 3-AT in the context of the X1–Y1 interactions. Last, the low levels observed could be due to positioning effects and/or sterical hindrance at the promoter of the reporter gene. In support of this hypothesis, the pairs X1–Y1 and X2–Y2, while having comparable stabilities, display different β-galactosidase activities. A difference between the two pairs is the introduction of one additional base pair in X2. We suggest that this additional base pair is responsible for a different sterical positioning of the activation domain relative to the promoter, thereby influencing reporter activity. It would therefore be interesting to monitor the effect of the stem length on the β-galactosidase activity. Since the correct folding of the RNA molecules is critical, we are currently examining the possibility of increasing the stability of the activation domain via elongation of the flanking stems and determining the optimal scaffold of the two RNA constructs to insert RNAs X and Y in such a way to obtain strongest reporter activation by introducing longer stems at the base of the hairpins.

The correlation between in vitro stabilities and reporter activation in the RNA-hybrid system implies that the results obtained in 1 M Na+ are relevant in vivo. The stability of the kissing loop complex is highly dependent on the ionic conditions (Weixlbaumer et al. 2004). Divalent magnesium ions have a great influence on the complex stability, and a specific magnesium ion binding site has been detected in the X-ray

FIGURE 5. Selection of interacting partners from a random library. (A) Sequences of clones obtained after selection using Y2 as bait. Sequences of the expected result (X2) and of the library are indicated. (B) Quantitative analysis of β-galactosidase activity of the different clones. The activity is normalized relative to the activity of X1–Y1 (1.0). Error bars indicate standard deviation. The library clones were tested together with Y1 (gray) and Y2 (white).
structure of the complex (Ennifar et al. 2001). Comparison of the kissing complex stabilities in the presence of Na⁺ or Mg²⁺ in vitro showed that 1 M Na⁺ results in similar stabilities to 0.5–1 mM Mg²⁺ (Weixlbaumer et al. 2004). Our results reported here indicate that these similarities are also valid in vivo. While secondary structure formation is less dependent on divalent ions, it is still surprising that 1 M Na⁺ might after all be a suitable ionic condition for the prediction of secondary and tertiary interactions in vivo.

Certainly with ~20,000 putative ncRNAs already present in the mammalian ncRNA database (Pang et al. 2005), the RNA-hybrid system, while requiring some further improvements, could become a useful tool in the investigation of the role of so many transcripts with unknown function by revealing their target genes. Alternatively, the system may also be used to screen for drugs inhibiting essential RNA–RNA interactions in pathogens such as the DIS-loop interaction in HIV.

MATERIALS AND METHODS

**Plasmid construction**

To construct plasmid m26-11 (Buskirk et al. 2003) two oligonucleotides were hybridized (sequences, 5'–ATCCCCGGGCCCCGCTGCATG-3' and 5'-GCCAGGATGC-AAAGGCTCGTGCCTTGCCCTGCAGG-3'). X1a to X1c and Y1a to Y1c were obtained with similar oligonucleotides. X50 and Y50 were cloned after PCR of pET3A-StpA (Zhang et al. 1995) dependent on divalent ions, it is still surprising that 1 M Na⁺ results in similar stabilities to 0.5–1 mM Mg²⁺ (Weixlbaumer et al. 2004). Our results reported here indicate that these similarities are also valid in vivo. While secondary structure formation is less dependent on divalent ions, it is still surprising that 1 M Na⁺ might after all be a suitable ionic condition for the prediction of secondary and tertiary interactions in vivo.

Certainly with ~20,000 putative ncRNAs already present in the mammalian ncRNA database (Pang et al. 2005), the RNA-hybrid system, while requiring some further improvements, could become a useful tool in the investigation of the role of so many transcripts with unknown function by revealing their target genes. Alternatively, the system may also be used to screen for drugs inhibiting essential RNA–RNA interactions in pathogens such as the DIS-loop interaction in HIV.

**Library construction**

The library was obtained after ligation in pAN MS2-2 of a PCR product (primers, 5'-CTAGTGGATCCCCGGGCGE-3' and 5'-CTGACGCTGCA CGGGCCG-3'; template, 5'-CTAGTGGATCC CGGGGGCGAANNNNNACGCCCCGTGCATGCCTGCAG-3') digested with SphI and Xmal.

**Yeast culture and transformation**

YBZ-1 yeast cells were cotransformed with plasmids derived from pIIta-m26 and plasmids derived from pACT MS2-2. pIIta-m26–derived plasmids carry the URA3 gene, and pACT MS2-2–derived plasmids contain the LEU2 gene. Transformed cells were selected on media lacking uracil and leucine (Bernstein et al. 2002).

Quantitative determination of β-galactosidase activity was performed by using o-nitrophenyl-β-D-galactopyranosidase (ONPG) as substrate as described in the Yeast Protocols Handbook from Clontech Laboratories (http://www.clontech.com).

For the comparative growth assay, serially diluted YBZ-1 strains transformed with a pACT MS2-2 and a pIIta-m26 derivative were plated on selective medium lacking uracil, leucine, and—when indicated, histidine—and supplemented with 3-AT.

For the screening, strain YBZ-1 was transformed successively with the plasmid expressing Y2 and with the library. Transformed cells were plated on medium lacking histidine and leucine and containing 0.2 mM 3-AT. After 4 d, 22 colonies were growing. The 10 white colonies were streaked on the same medium. Colonies remaining white were streaked on medium lacking leucine and containing 5-fluorotic acid (5-FOA). Plasmids from active clones were recovered by using standard techniques (Sambrook and Russell 2001) and sequenced with primer pIIIa 4827 (5'-CTGTATCGAAATAAAGTGA-3').

**Northern blot**

For Northern blotting, transformed yeast cells were grown to mid-log phase (OD600 0.8) and RNA was prepared by using the RNaseasy Mini Protocol for Isolation of Total RNA from yeast (enzymatic lysis protocol, standard version) from Qiagen; 25 μg of the RNA samples were denatured and loaded on a 5% denaturing polyacrylamide gel before semi-dry transfer (Lee et al. 1993). The PCR probe from m26-11 (using oligonucleotides plIIta rev 5162 5'-CTCTATACTCCCCCTATAGCTGC-3' and plIIta 4827) was labeled by random priming. The probe was hybridized according to a standard protocol (Sambrook and Russell 2001).
ACKNOWLEDGMENTS

We thank Prof. Marvin Wickens for the gift of the three-hybrid system strain and plasmids. We thank Stefan Ameres and Christina Lorenz for helpful discussions, and all of the Schroeder laboratory for critical review of the manuscript. This work was supported by FWF (Austrian Science Foundation) grant no. Z-72.

Received May 9, 2005; accepted September 24, 2005.

REFERENCES


A yeast RNA-hybrid system for the detection of RNA–RNA interactions in vivo
NICOLAS PIGANEAU, URSULA E. SCHAUER and RENÉE SCHROEDER
RNA 2006 12: 177-184

References
This article cites 25 articles, 4 of which can be accessed free at:
http://rnajournal.cshlp.org/content/12/1/177.full.html#ref-list-1

License

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.