Mechanism of Dimerization of Bicoid mRNA

INITIATION AND STABILIZATION*

Céline Wagner†, Chantal Ehresmann, Bernard Ehresmann, and Christine Brunel‡

From the Institut de Biologie Moleculaire et Cellulaire, Unité Propre de Recherche 9002 du CNRS, 15 rue René Descartes, 67084 Strasbourg Cedex, France

Received for publication, June 19, 2003, and in revised form, November 6, 2003
Published, JBC Papers in Press, November 7, 2003, DOI 10.1074/jbc.M306511200

Dimerization of bcd mRNA was shown to be important for the formation of ribonucleoprotein particles and their localization in Drosophila embryo. The cis-element responsible for dimerization is localized in a stem-loop domain (domain III) containing two essential complementary 6-nucleotide sequences in a hairpin loop (LIIIb) and an interior loop (LIIIa). Such an RNA element can potentially generate single or double “hand-by-arm” interactions leading to open and closed complexes, respectively. The former retains the possibility of forming multimers, whereas the latter does not. We showed previously that dimerization proceeds through a two-step mechanism, which includes a transition from the reversible initiation complex into a very stable one. Here we have addressed the nature of the initial interactions and the mechanism of transition. We engineered a series of different RNA fragments with the capacity to form defined open dimers, multimers, or closed dimers. We compared their thermodynamic and kinetic behavior and mapped nucleotides involved in intermolecular interactions by enzymatic and chemical footprinting experiments and chemical modification interference. Our results indicate that the initiation step leads to a reversible open dimer, involving a more limited number of intermolecular base pairs than expected. The two loops play distinct roles in this process, and the structure of loop IIIb is more constrained than that of loop IIIa. Thus, loop IIIa appears to be the driving element of the recognition process. The initial open dimer is then converted into a stable closed dimer, possibly through a kinetically controlled mechanism.

RNA loop-loop interactions are commonly used to trigger initial recognition between two RNA molecules and to modulate a high diversity of biological functions (for a review, see Ref. 1). The frequent selection of hairpin loops as recognition motifs is explained by their intrinsic properties. Their accessibility and structural versatility allow them to adopt a variety of conformations, thus enabling an appropriate presentation of nucleotides that initiate the recognition process. This initiation step generally involves Watson-Crick pairing of a few nucleotides, preferentially G-C pairs. Usually, efficient recognition requires rapid bimolecular binding rates, regardless of the RNA pairing scheme. The initial loop-loop complex generally serves as an intermediate for subsequent stabilization involving different possible paths, such as isomerization, helix propagation, and formation of additional interactions (1–3).

Hairpin loops recognition is particularly well documented in the case of natural antisense RNAs, which play a variety of regulatory roles in bacteria and their extrachromosomal elements (1, 4, 5). Another well studied case is the dimerization of genomic RNA of HIV-1,1 which is initiated by a loop-loop interaction involving an autocomplementary six-base sequence in a hairpin loop (reviewed in Ref. 1). The above mentioned examples mainly concern recognition between two hairpin loops and are referred to as “kissing” interactions. Interaction might also involve complementary sequences located in a hairpin loop and an interior loop (“hand-by-arm” interaction). This type of interaction can potentially generate “closed” dimers, involving a double symmetric hand-by-arm interaction, or multimers. The latter mode is used by Bacillus subtilis bacteriophage phi29 RNA, in which cyclic hexamers of the 120-base pro-head RNA are formed and are needed for efficient in vitro packaging of the DNA genome (6, 7). Another example of hand-by-arm interaction is provided by dimerization of bicoid (bcd) mRNA, the product of which specifies the head and thorax pattern in Drosophila melanogaster embryos.

Dimerization of bcd mRNA was shown to be important for the formation of ribonucleoprotein particles containing bcd mRNA and their localization in the embryo (8, 9). The cis-acting element that triggers dimerization is part of the 3′-UTR of the mRNA, which folds into five well defined domains (Fig. 1E).2 It corresponds to an RNA stem loop (domain III) that contains six-base complementary sequences located in the apical loop, LIIIb, and the interior loop, LIIIa (Fig. 1D). Recently, we showed that bcd mRNA dimerization is a two-step mechanism involving a rapid conversion of the initial reversible loop-loop complex into an almost irreversible one (9). This was supported by dissociation kinetics and the observation that sense or antisense oligonucleotides are unable to inhibit dimerization once stabilization has begun. On the other hand, we found that the isolated domain III (RNA III) formed reversible dimers and multimers, as accounted for by the hand-by-arm interaction possibilities, whereas the complete 3′-UTR RNA did not. It appeared from deletion analysis that domain I and the major part of domains IV and V are dispensable for the transition to the stable complex (9). This raises two important questions: (i) is a single or a double hand-to-arm interaction

* This work was supported by National Institutes of Health Grant GM54447. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a fellowship from “La Ligue contre le Cancer.”
‡ To whom correspondence should be addressed. Tel.: 33-388417040; Fax: 33-388602218; E-mail: C.Brunel@ibmc.u-strasbg.fr.

1 The abbreviations used are: HIV-1, human immunodeficiency virus 1; UTR, untranslated region; DMS, dimethylsulfate; DEPC, diethylpyrocarbonate; ENU, ethylnitrosourea; CMCT, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate; Do, open dimer; De, closed dimer.
2 C. Brunel and C. Ehresmann, submitted for publication.
required for initiating dimerization, and (ii) how does stabilization proceed?

In the present work, we constructed RNA fragments of different lengths, which can form either two or a single hand-by-arm interaction, and we compared their ability to be converted into stable complexes. We used enzymatic and chemical probing to map the interactions in the different kinds of complexes. Finally, we used chemical modification interference of the phosphate backbone and bases to identify individual positions of nucleotides that are essential in unmodified form to the dimerization process. Our results provide important clues on both the initial step of dimerization and the stabilization pathway. They also highlight the importance of loop IIIb structure as a trigger of the initial recognition and of the structural context in the fate of the reversible dimer.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and RNA Synthesis**

Plasmids used in this study were described previously (9). The NlaIV-HindIII bcd fragment of the plasmid pAI(V+V) (9) was subcloned into the StuU-HindIII sites of the pUT7 vector, a pUC derivative containing the T7 RNA polymerase, to create the plasmid pAI(1+1+V). PCR fragments corresponding to the isolated domain III (m,w) or (w,m) were generated from plasmid 875 (m,w) using primers 1 and 2 and from plasmid 875 (w,m) using primers 1 and 3 (Table I). RNA III issued from those PCR products starts at position 231 and ends at position 335 (using the numbering of the full-length bcd 3 ’UTR). The III′ series starting at position 231 and ending at position 347 was generated from the plasmid 875 (m,w) using primers 1 and 4 and from plasmid 875 (w,m) using primers 1 and 5. PCR products were purified by gel electrophoresis and transcribed by phage T7 RNA polymerase (10). Transcription with incorporation of ApG at the 5 ’terminus of the transcripts allowed efficient 5 ’end-labeling by T4 polynucleotide kinase with [γ-32P]ATP (10). Unlabeled and labeled RNAs were purified by polyacrylamide-urea gel electrophoresis, eluted overnight with 0.5 M sodium acetate, 10 mM EDTA, pH 8.0, 0.2 volume of phenol, ethanol-precipitated, and dissolved in water.

**Dimerization of RNAs**

RNAs were heated for 3 min at 85 °C and cooled slowly at 60 °C. After the addition of the 5-fold concentrated appropriate buffer, the samples were incubated for 30 min at 25 °C and then chilled on ice. Standard dimerization buffer (D1) contained 50 mM sodium cacodylate, pH 7.5, 75 mM KCl, 5 mM MgCl2. Monomers were obtained by replacing buffer D1 by buffer M1 (50 mM sodium cacodylate, pH 7.5, 50 mM KCl, 0.1 mM MgCl2). When necessary, monomeric and dimeric forms were separated by gel electrophoresis. Short fragments (RNA III series) were fractionated at 4 °C on 10% acrylamide/bis-acrylamide 1/30) gels in 45 mM Tris borate, pH 8.3, 50 mM MgCl2, and larger fragments on 1% agarose gel in the same buffer.

**Kd Determination and Derived Procedures**

The apparent dissociation constants (Kd) were evaluated by mixing a negligible constant concentration of labeled wild-type RNA III (about 1 nM) with increasing concentrations of its unlabeled RNA partner (ranging from 4.6 to 270 mM). Dimerization was conducted under standard conditions in buffer D1 and fractionated by electrophoresis (see above). Radioactivity was measured with a BAS 2000 Bio-Imager. Kd values were estimated as the concentration of unlabeled RNA necessary to obtain half-saturation, assuming that complex formation (homo- or heterodimers) obeys a simple bimolecular equilibrium. The treatment assumes that [free unlabeled RNA] = [total unlabeled RNA]. This condition is fulfilled in the assay because the concentration of labeled wild-type RNA III is negligible compared with the concentration of its unlabeled RNA partner. Kd values were the average of at least three independent experiments and have a maximum error of ±50%. For kinetics of association, a negligible constant concentration of labeled wild-type RNA III was mixed with a constant concentration (50 nm and 100 nm) of its unlabeled RNA partner in buffer D1, and aliquots were loaded onto the gel at different times (9). For dissociation experiments, the complex was formed at equilibrium (a concentration that favours multimerization of wild-type RNA III), and subjected to a 50-fold dilution. Aliquots were analyzed as a function of time (9). Time 0 was obtained by running an aliquot of the dimerization mixture immediately before dilution. It was also verified that no dimer was formed when the RNA was incubated for 30 min at the final concentration obtained after dilution.

**Probing and Footprinting Experiments**

Most experiments were conducted on 5 ‘end-labeled fragments (1.2 μm) of the RNA III series.

**Enzymatic Hydrolyses**—Incubation was for 2 to 8 min at 25 °C in the presence of 0.2 unit of RNase T1, 0.02 unit of RNase T2, and 0.2 or 0.05 unit of RNase V1 in buffer D1 or M1. Reactions were stopped by phenol/chloroform extraction followed by ethanol precipitation. Dimethylsulfate (DMS) Modification (C/N3)—Reaction was for 5 min at 25 °C with 2.5 or 5 μl of DMS in buffer M1 or D1. After precipitation with 0.3 M sodium acetate and 3 volumes of ethanol, samples were treated with 10 μl of hydrazine 10% for 5 min at 0 °C and precipitated. DMS modification with ENU was for 1 min at 90 °C with 0.3 M sodium acetate and 3 volumes of ethanol, samples were treated with 10 μl of hydrazine 10% for 5 min at 0 °C precipitated. The modified RNAs were then incubated with 10 μl of aniline for 10 min at 60 °C for the cleavage reaction. Cleavage products were analyzed by gel electrophoresis on a 10% polyacrylamide/8 M urea gel. RNase T1 and alkaline ladder of end-labeled RNAs were run in parallel to facilitate band assignment (11). In the case of RNase T1 footprinting on larger fragments (RNA II series), cleavage was detected by reverse transcriptase extension of a primer complementary to nucleotides 389–400. Fractionation was on 10% polyacrylamide/8 M urea gel.

**Phosphate Modification Interference**

The appropriate 5 ‘end-labeled fragment of the RNA III series (0.35 μm) was alkylated on its phosphate groups by ethylnitrosourea (ENU). Modification was for 1 min at 90 °C with 0.5 volume of ENU-saturated ethanol in 50 mM sodium cacodylate, pH 6.5, 50 mM KCl, 1 mM EDTA, in the presence of total tRNA (2 μM) as carrier. An incubation control without the ENU was done in parallel using the same conditions except that ENU was omitted. The reaction was stopped by precipitation with 0.3 M sodium acetate and 3 volumes of ethanol. The modified RNA was then dissolved in water, mixed with its unlabeled partner, and submitted to the dimerization procedure. Dimeric and monomeric species were fractionated and RNAs eluted from the gel by overnight incubation at 4 °C in 0.5 M sodium acetate, 10 mM EDTA, pH 8.0, 0.2 volume of phenol. After ethanol precipitation, ethylated phosphates were cleaved by incubation in 10 μl of Tris-HCl 0.1 M, pH 9.0, at 50 °C for 10 min. End-labeled RNA fragments were sized on a 10% polyacrylamide/8 M urea gel. Cleavage positions were identified by running in parallel the RNase T1 and alkaline ladders of the end-labeled RNA (11).

**Modification Interference at Watson-Crick Positions**

The appropriate fragment (1.2 μm) of the RNA III series was modified with DMS (A/N1) and C/N3) or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMCT) (G/N1) and U/N3)). Modification with DMS was for 1 min at 90 °C with 0.001 volume of DMS in 50 mM sodium cacodylate, pH 7.5, 50 mM KCl, 1 mM EDTA in the presence of total tRNA (2 μM) as carrier. Modification was stopped by phenol/chloroform extraction (100 μl) and ethanol precipitation. Modification with CMCT was for 1 min at 90 °C with 0.025 volume of CMCT (70 mg/ml) in 50 mM sodium borate, pH 7.5, 50 mM KCl, 1 mM EDTA in the presence of total tRNA (2 μM) as carrier. Modification was stopped by ethanol precipitation. Incubation controls were run in parallel in the absence of chemicals. The modified RNAs were mixed with their unmodified partner and subjected to the dimerization procedure. Dimeric and monomeric species were fractionated as above. In this case, modified nucleotides were analyzed by primer extension with reverse transcriptase, using as a primer an oligonucleotide complementary to nucleotides 335–347. Conditions were adapted from Brunel et al. (12).
RESULTS

RNA III Preferentially Forms One Hand-to-Arm Interaction—We previously showed that wild-type RNA III (RNA III(w,w)) forms dimers that dissociated rapidly, whereas larger tested RNAs do not dissociate within 1 h (9). In addition, RNA III multimers were formed in a concentration-dependent manner and dissociated rapidly. RNA III(w,w), which contains both loops LIIIa and LIIIb available for interactions, can form either a double hand-by-arm interaction (closed dimer, Fig. 1A) or single interactions leading to multimers (Fig. 1B). Because the initial recognition of bcd mRNA can potentially be driven by one or two hand-to-arm interactions, we compared the intrinsic behavior of RNA III(w,w) and derive variants mutated in one of the two loops. Mutations introduced were identical to those already used (8, 9) (Fig. 1D). RNA III(m,w) carried the substitution of AGUGAC for AAGCCC282 in loop LIIIb and a wild-type sequence loop LIIIa. Conversely, RNA III(w,m) carried the substitution of GUCACU for GGGCUU322 in loop LIIIa and a wild-type sequence loop LIIIb. Each mutated RNA was unable to dimerize by itself. However, the addition of RNA III(w,w) permitted the recruitment of the mutant RNA in a heterodimer through one hand-to-arm interaction (Fig. 1C, “open” dimer).

This system allowed us to evaluate the apparent dimerization dissociation constant \( K_d \) of different combinations of RNA III. Experiments were done by mixing a constant negligible amount of labeled wild-type RNA III(w,w) with increasing concentrations of unlabeled RNA III(w,m) or RNA III(m,w). Because RNA III(w,w) is unable to dimerize at the chosen concentration, it was possible to study the properties of open dimers exclusively. The \( K_d \) of RNA III(w,w), which could not be determined precisely, because of the formation of multimers, was estimated to be approximately 10 nM. The \( K_d \) values determined for both heterodimers formed with either RNA III(m,w) or (w,m) fell in the same range (Fig. 2C).

Then we compared association and dissociation of the complexes formed by RNA III(w,w) alone or in association with RNA III(w,m) or III(m,w), as a function of time. The formation of wild-type dimers and heterodimers was already completed within 30 s of incubation at both 50 and 100 nM (Fig. 2A). This rapid association contrasts with the slower association observed for the complete 3′-UTR for which half-association required 12 min at a concentration of 125 nM (9). As expected, trimers and low amounts of higher species were also observed in the case of RNA III(w,w). For dissociation experiments, the complex was formed at 5 nM (a concentration that disfavors multimerization of wild-type RNA III) and subjected to a 50-fold dilution (9). Half-dissociation occurred within the minute range in every case (Fig. 2B), again contrasting with an absence of dissociation in the case of the complete 3′-UTR (9). However, the heterodimers were totally dissociated, whereas the wild-type RNA yielded a constant fraction (30%) of dimer resistant to dissociation (Fig. 2B). This finding suggests that RNA III(w,w) forms two types of dimers. The major fraction, which displays kinetic properties similar to the heterodimers,
probably corresponds to dimers utilizing a single loop-loop interaction (open dimers). These dimers are available to further multimerization. The minor fraction that forms stable dimers might correspond to closed dimers.

Probing the Conformation of RNA III and Its Two Variants—We probed the conformation of monomeric RNA III (w,m) and III (m,w) with enzymatic and chemical probes. RNase T1 cleaves unpaired G residues, RNase T2 preferentially cleaves unpaired As (and Us to a weaker extent), and RNase V1 cleaves double-stranded regions. DMS and DEPC alkylate C(N3) and A(N7), respectively. RNA III (w,m) was probed under low salt conditions (buffer M1), which favor the monomeric form. RNA III (w,m) and III (m,w), which are intrinsically unable to self-dimerize, were tested under the same conditions but also under the high ionic conditions (buffer D1) used to promote dimerization. Representative gels are shown in Fig. 3, and the results obtained for the three RNAs are summarized in Fig. 4A.

The experimental data fully account for the proposed secondary structure shown in Fig. 4A. In particular, loops IIIa and IIIb were highly accessible to enzymes and chemical reagents in the three RNA tested. The cleavage and modification profiles of the three RNAs looked very similar, with only a few changes at or close to the mutation sites. The expected changes directly linked to the mutations are observed (e.g. new RNase T1 cut at G278 and G280 instead of G279 in RNA III (m,w), disappearance of cleavage at G318 and G319 in RNA III (m,w)), whereas G278 and G280 remained fully accessible in the mutated loop IIIb. Conversely, G279 was protected in RNA III (w,m) upon association with RNA III (w,m), whereas G317 remained accessible. Notably, cleavage at G309 and G310, which was enhanced as a result of loop IIIa mutation (see above), was reduced upon formation of the heterodimer with RNA III (w,m) (Fig. 3A). This protection might result from a stabilization induced by the high ionic concentration used for dimerization rather than dimerization itself. Homodimers were formed by mixing 5’-labeled RNA III (w,m) and unlabeled RNA III (w,m), so that only the RNA forming one loop-loop interaction could be visualized. A typical experiment is shown in Fig. 3A, and the results are summarized in Fig. 4B. Upon its association with RNA III (w,m), RNA III (m,w) underwent a loss of cleavage at G317, G318, and G319, whereas G278 and G280 remained fully accessible in the mutated loop IIIb.

Footprinting Loop-Loop Interactions in RNA III Complexes—The involvement of loop IIIa and IIIb nucleotides in intermolecular interactions was first tested by RNase T1 footprinting in the three types of complexes. Heterodimers were formed in buffer D1 by mixing 5’-labeled RNA III (w,m) or III (m,w) and unlabeled RNA III (w,m), so that only the RNA forming one loop-loop interaction could be visualized. A typical experiment is shown in Fig. 3A, and the results are summarized in Fig. 4B. Upon its association with RNA III (w,m), RNA III (m,w) underwent a loss of cleavage at G317, G318, and G319, whereas G278 and G280 remained fully accessible in the mutated loop IIIb.

Conversely, G279 was protected in RNA III (w,m) upon association with RNA III (w,m), whereas G317 remained accessible. Notably, cleavage at G309 and G310, which was enhanced as a result of loop IIIa mutation (see above), was reduced upon formation of the heterodimer with RNA III (w,m) (Fig. 3A). This protection might result from a stabilization induced by the high ionic concentration used for dimerization rather than dimerization itself. Homodimers were formed by mixing 5’-labeled RNA III (w,m) and unlabeled RNA III (w,m). A clear protection in both loops (G279 in loop IIIb and G317, G318, and G319 in context. Nonetheless, this is a unique reactivity change not directly located at mutation sites, and taken together the data show that mutations do not perturb the structural organization of the RNA. In addition, probing the RNA III variants at both salt conditions did not reveal any significant difference (data not shown). Finally, cleavage and modification patterns were very similar to those obtained for wild-type or mutated domain III in the full-length bcd 3’-UTR,2 leading to the conclusion that domain III forms an independent structural domain and that RNA III is representative of what happens in the whole RNA.
Fig. 3. Probing and footprinting experiments. Hydrolysis or chemical modification was performed on unlabeled 5’ end-labeled RNA III (w,w), (m,w), or (w,m) (denoted by an asterisk) in the absence or presence of unlabeled RNA III(w,w) under low (L) or high ionic conditions (H). The positions of loops IIIa and IIIb are indicated. A, RNase T1 hydrolysis of the RNA III series, for 2 and 4 min (lanes 1 and 2, respectively). Lane C is an incubation control in the absence of RNase T1. Lanes L and T1, alkaline and RNase T1 ladders, respectively. B, modification of C(N3) by DMS. Incubation was for 5 min in the absence (lane C) or presence of 1 and 5 µl (lanes 1 and 2, respectively) of DMS. C, modification of A(N7) by DEPC. Incubation was for 12 min in the absence (lane C) or presence of 2.5 and 5 µl (lanes 1 and 2, respectively) of DEPC.
loop IIIa was observed, as expected from the implication of both loops in multimers (Fig. 1B).

We then used DMS and DEPC to get a more precise footprint of the loop-loop interactions and to test the participation of the two 6-nucleotide complementary sequences (GGGCUU in loop IIIa and AAGCCC in loop IIIb) in intermolecular base pairing. Modification with these two chemicals, followed by direct detection on end-labeled RNAs, allows probing of cytosine at a Watson-Crick position (N3) and adenine at its Hoogsteen position (N7), respectively. Note that the nonreactivity of

![Diagram](https://via.placeholder.com/150)
A(N7) can be caused either by direct involvement in hydrogen bonding or by base stacking (13). As expected, a strong reduction of reactivity of C280, C281, and C282 to DMS was observed in RNA III(w,m) upon association with RNA III(w,w) as well as in RNA III(w,w) multimers (Figs. 3B and 4B). This result accounts for base pairing between these three C residues and complementary G residues (317–319) of loop IIIa. Besides, the reactivity of C274 was unchanged. More unexpectedly, C318 remained fully reactive to DMS (even a little more) in RNA III(m,w)/RNA III(w,w) heterodimers and in RNA III(w,w) multimers (Figs. 3B and 4B). This result argues against base pairing between C318 and G279, suggesting that base pairing is more limited than expected, at least in the open complexes.

Chemical Modification Interference—Chemical modification interference was used to obtain more information about the functional groups required in the unmodified form for initial recognition. In these experiments, 5’ end-labeled RNA III with the different loop combinations was submitted to limited modification (less than one statistically distributed modification/molecule) under denaturing conditions and subjected to the dimerization procedure in the presence of unmodified and unlabeled RNA III(w,w). Dimerization-competent molecules and noncompetent molecules were fractionated by polyacrylamide gel electrophoresis, purified, and analyzed. Modifications present in the monomeric species but absent from the dimeric one are those that negatively interfere with dimerization. Conversely, modifications that are more abundant in the dimeric than in the monomeric form positively influence dimerization. Representative experiments are shown in Fig. 5, and the results are summarized in Fig. 6.

First, we used ethynitrosourea (ENU) interference to map nonbridging oxygens of the phosphate groups, which are required to be unmodified for initial recognition (Fig. 5AB). The analysis of the single loop-loop interaction between labeled modified RNA III(w,m) and unlabeled RNA III(w,w) revealed a single window of negative interference, corresponding to complete inhibition of dimerization upon ethylation of any phosphate group 3’ to nucleotides 277–284. A weak but reproducible positive interference was also observed at phosphates 273–275. These A-residues were highly reactive in the free RNA, it appears that the stacked conformation results from a conformational adjustment triggered by formation of the limited intermolecular interactions.

Fig. 5. Chemical modification interference. A, phosphate ethylation interference by ENU. Modification was conducted on 5’ end-labeled RNA III(w,w), (m,w), or (w,m) as indicated at the top of the gel. Dimerization was then allowed with unlabeled RNA III(w,w), and the resulting monomers and dimers were fractionated by gel electrophoresis. B and C, CMCT and DMS interference. The same protocol was used as described in A, except that the tested RNA III(w,m) was unlabeled, and modified positions were revealed by primer extension. M and D, RNA extracted from the monomer and the dimer bands, respectively; T, total population of modified RNA. C, incubation control. Lanes L and T correspond to alkaline and RNase T1 ladders, respectively. Negative interference is indicated by arrowheads. A complete or almost complete disappearance of a band revealed a “strong” interference (black arrow), and only a slight decrease indicated a “weak” interference (gray arrow). Positive interference is denoted by an asterisk.

Second, we used ethynitrosourea interference to map nonbridging oxygens of the phosphate groups, which are required to be unmodified for initial recognition (Fig. 5AB). The analysis of the single loop-loop interaction between labeled modified RNA III(w,m) and unlabeled RNA III(w,w) revealed a single window of negative interference, corresponding to complete inhibition of dimerization upon ethylation of any phosphate group 3’ to nucleotides 277–284. A weak but reproducible positive interference was also observed at phosphates 273–275. On the other hand, the interaction between loop IIIa of RNA III(m,w) with loop IIIb of RNA III(w,w), was strongly reduced by ethylation of phosphate groups 3’ to nucleotides 317–324. ENU interference was also tested on RNA III(w,w). Much weaker interference windows could be detected in loops IIIa and IIIb, coinciding with those observed in the open heterodimers (Fig. 5, A and B). The absence of strong interference in the wild-type RNA was not surprising, because the modifi-
Initiation of Bicoid mRNA Dimerization

Initial Recognition Involves Only a Limited Number of Nucleotides—Here we show that the isolated domain III (RNA III) provides a valuable model to understand the first steps of dimerization of the complete bed 3′-UTR. We used different combinations of wild-type RNA III and variants in one of the two loops with the capacity to form either open or closed interactions. We found that wild-type RNA III preferentially forms open reversible interactions, thus allowing formation of multimers that increase with RNA concentration. Notably, multimers and open heterodimers were undistinguishable, insofar as their thermodynamic and kinetic properties, as well as their probing and footprinting profiles, are concerned. One unexpected result is that base pairing between the two loops is not as extended as believed previously. Indeed, the chemical foot-
print indicated that on the six potential base pairs (AAGCC\(^{282}\)/GGGCUU\(^{282}\)), only three are stably formed (CCC\(^{282}\)/GGGG)\(^{15}\). This is reminiscent of antisense RNAs, in which initial recognition involves only a limited number of nucleotides (for review see Ref. 1). The identified initial interaction involves G-C pairs, a feature often encountered in the loops of natural antisense RNAs that trigger recognition. Notably, the dimerization initiation site of HIV-1 genomic RNA contains a six-base complementary sequence with a conserved central GC, a feature that was also selected among degenerated pools of RNAs capable to homodimerize (14). These observations suggest that G-C base pairs are frequently used as the nucleation point of loop-loop interactions.

The Two Loops Play Distinct Roles—The most striking information provided by interference experiments is the unbalanced response of loops IIIa and IIIb. Indeed, loop IIIb appeared much more sensitive to modification than loop IIIa. The large window of interference to both phosphate ethylation and base modification covers the greater portion of loop IIIb, for the most part exceeding the nucleotides involved in the initial base pairing, whereas positive interference was observed in the 5’ part of the loop. Such a high susceptibility to phosphate ethylation appears to be a common theme in loop-loop interactions (i.e. the loops of the antisense RNA CopA and its target CopT (2)). A similar ethylation interference pattern (with negative interference in most part of the loop and positive interference in the 5’ part) was observed in the 9-nucleotide loop of the HIV-1 dimerization initiation site containing the 6-nucleotide self-complementary sequence (10). However, in these systems, base modification interference was restricted to nucleotides involved in initial pairing. Such an extent of interference by DMS and CMCT was rather unexpected and likely suggests that small perturbations of the loop topology are sufficient to inhibit loop-loop recognition. By contrast, loop IIIb appeared poorly, if at all, sensitive to DMS and CMCT modification, whereas interference by ENU was observed at discrete positions.

These differences highlight the different structural constraints and specific roles of the two loops in initial recognition. Thus, loop IIIb should adopt a precise and unique topology that is required for recognition of loop IIIa, which in turn does not appear to be structurally constrained. Most likely, the only requirement for loop IIIa recognition is to be accessible and sufficiently flexible to be available for base pairing with bases displayed by loop IIIb. A similar mode of recognition governs the codon-anticodon interaction, in which the structure of the tRNA anticodon loop (and not the number of base pairs) plays a crucial role in correctly positioning bases in a pre-formed A helical conformation (15). This holds also true for antisense/target RNA recognition, where the proper conformation of loops is required for initial recognition. Notably, the target or antisense loop generally contains a conserved YUNR motif proposed to form a U-turn motif similar to that of the anticodon loop (16, 17). Thus, we can assume that structurally not yet identified features, such as noncanonical interactions and ion binding, are required for correct presentation of nucleotides.
Initiation of Bicoid mRNA Dimerization

Stabilization Involves Conversion of Open to Closed Interactions—The next question concerns the nature of the elements that trigger the conversion. We first hypothesized that sequences in domain II, or in the large hinge connecting domains II, III and IV, might be involved in this process (9). However, large deletions in the hinge region (or replacements by stable stem loops) did not alter the capacity to promote stabilization (results not shown). Furthermore, no conserved sequences were found in these regions. The observation that a fraction of wild-type RNA III yielded stable dimers, whereas forced open heterodimers could not, was a first indication that stabilization could be triggered by formation of closed interactions (this work). Thus, we constructed an RNA fragment lacking domains I, IV, and V, which were previously showed to be dispensable for stabilization. Importantly, this RNA formed very efficiently stable dimers, while unable to form multimers. Then we showed, using RNase T1 footprinting, that both loops IIIa and IIIb were engaged in closed interactions in the stabilized dimer. This was the first experimental evidence for the actual existence of the double hand-to-arm interaction in a dimer. Our results also indicate that formation of the single loop interaction is rapid and reversible in the isolated RNA III (this work), whereas larger fragments form dimers much slower (9). Indeed, half-association was reached in 2.5 and 12 min for RNA ΔI and RNA 875′, respectively, at a concentration of 125 nM (9), whereas it was completed in less than 30 s for RNA III at 50–100 nM.

Taken together, our findings provide new insight into the two-step mechanism, allowing us to propose the following model. The first step involves the recognition of a few nucleotides in loop IIIa of one molecule by loop IIIb of another molecule, leading to a reversible open dimer (Do). The following step corresponds to the conversion of Do into a stable closed dimer (Dc), with both loops engaged in symmetrical hand-to-arm interaction. Our data suggest that the mechanism of the Do to Dc transition may be controlled kinetically (Fig. 8). It can reasonably be assumed that the first step (formation of Do) would obey a bimolecular association, depending on concentration and diffusion. Otherwise, the conversion of Do into Dc can be assimilated to a monomolecular reaction, independent of RNA concentration (Fig. 8). Thus, the fate of Do (multimerization or stabilization) would be driven by the association rate of the two steps. If the association rate of the formation of Do (k + 1) is higher than the rate of conversion of Do into Dc (k + 2), the reaction would be displaced toward multimer formation. Conversely, if k + 2 is higher than k + 1, the reaction would tend toward the monomolecular conversion. This situation might occur when k + 1 decreases as the length of RNA increases. Although this simplified model describes most of our observations, further experiments will be required for testing its validity (i.e., by precise determination of rate constants using more accurate techniques).

Whether the conversion of the initial open dimer, Do, into the stable closed dimer, Dc, is accompanied by an extension of base pairing is presently unknown. Indeed, the reactivity of C320(N3), which was still very high in open dimers of RNA III (this work), could not be tested in larger RNAs due to reverse transcriptase pausing, whereas G279, the putative partner of C320, was unreactive in both monomer and dimer species. Otherwise, one might assume that additional interactions (i.e., helix/helix or bulge/helix) also contribute to the high stability of the dimer. Further investigation will be required to answer this question.

Thus, bcd mRNA has developed a highly tuned mechanism to dimerize. Although the role of dimerization is still unclear, one might assume that one goal is to generate an intrinsic duplication of the numerous regulatory sites that are present in the 3′-UTR (18) or to create a new cis-acting element. The mechanism proposed from this study differs from that described for the prohead RNA of phage phi29, although they both utilize hand-to-arm interactions. The phi29 RNA does not form closed dimers but is used to build very stable hexamers that are held together by open interactions involving four complementary nucleotides in the lateral and apical loops (6, 7). One similar point might concern the first recognition step, which results, in both cases, in the formation of open dimers. However, although the bcd mRNA dimer is spontaneously converted into a stable dimer by the formation of a closed interaction, the phi29 RNA dimer is driven to the multimerization reaction, which stops at the level of hexamers, probably because of structural constraints. Clearly, this example highlights how similar recognition features can be used to achieve different biological purposes.

Acknowledgments—We thank F. Jossinet, E. Westhof, L. Jaeger, C. Isel, P. Romby, and R. Marquet for fruitful discussions. Flore Winter is acknowledged for skilful technical assistance.

REFERENCES
1. Brunel, C., Marquet, R., Romby, P., and Ehresmann, C. (2002) Biochimie (Paris) 84, 925–944
2. Köl, F. A., Engdahl, H. M., Slagter-Jager, J. G., Ehresmann, B., Ehresmann, C., Westhof, E., Wagner, E. G., and Romby, P. (2000b) EMBO J. 19, 5905–5915
3. Rist, M., and Marino, J. (2001) Nucleic Acids Res. 29, 2401–2408
4. Wagner, E. G., and Simons, R. W. (1994) Annu. Rev. Microbiol. 48, 713–742
5. Zeiler, B. N., and Simons, R. W. (1998) in RNA Structure and Function (Simons, R. W., and Grunberg-Manago, ed.) pp. 437–464, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
6. Chen, C., Sheng, S., Shao, Z., and Guo, P. (2000) J. Biol. Chem. 275, 17510–17516
7. Chen, C., Zhang, C., and Guo, P. (1999) RNA 5, 805–818
8. Ferrandon, D., Koch, I., Westhof, E., and Nussinov-Volhard, C. (1997) EMBO J. 16, 1751–1758
9. Wagner, C., Palacios, I., Jaeger, L., St Johnston, D., Ehresmann, B., Ehresmann, C., and Brunel, C. (2001) J. Mol. Biol. 312, 511–524
10. Jossinet, F., Paillart, J. C., Westhof, E., Hermann, T., Skripkin, E., Lodmell, J. S., Ehresmann, C., Ehresmann, B., and Marquet, R. (1999) RNA 5, 1222–1234
11. Brunel, C., and Romby, P. (2000) Methods Enzymol. 318, 3–21
12. Brunel, C., Romby, P., Westhof, E., Ehresmann, C., and Ehresmann, B. (1991) J. Mol. Biol. 211, 293–308
13. Ehresmann, C., Baudin, F., Mougel, M., Romby, P., Ellb, J. P., and Ehresmann, B. (1987) Nucleic Acids Res. 15, 9109–9128
14. Lodmell, J. S., Ehresmann, C., Ehresmann, B., and Marquet, R. (2000) RNA 6, 1267–1276
15. Grosjean, H., Marquet, R., and Romby, P. (1998) in Modification and Editing of RNA (Grosjean, H., and Benne, R., eds.) pp. 113–135, ASM Press, Washington, D. C.
16. Franch, T., Petersen, M., Wagner, E. G., Jacobsen, J. P., and Gerdes, K. (1999) J. Mol. Biol. 294, 1115–1125
17. Asano, K., and Mizobuchi, K. (2000) J. Biol. Chem. 275, 1269–1274
18. Maclonald, P. M., and Kerr, K. (1997) RNA 3, 1413–1420
Mechanism of Dimerization of Bicoid mRNA: INITIATION AND STABILIZATION
Céline Wagner, Chantal Ehresmann, Bernard Ehresmann and Christine Brunel

J. Biol. Chem. 2004, 279:4560-4569.
doi: 10.1074/jbc.M306511200 originally published online November 7, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306511200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 16 references, 8 of which can be accessed free at http://www.jbc.org/content/279/6/4560.full.html#ref-list-1