

# Role for gene looping in intron-mediated enhancement of transcription

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**Intron-containing genes are often transcribed more efficiently than nonintrinsic genes. The effect of introns on transcription of genes is an evolutionarily conserved feature, being exhibited by such diverse organisms as yeast, plants, flies, and mammals. The mechanism of intron-mediated transcriptional activation, however, is not entirely clear. To address this issue, we inserted an intron in *INO1*, which is a nonintrinsic gene, and deleted the intron from *ASC1*, which contains a natural intron. We then compared transcription of *INO1* and *ASC1* genes in the presence and absence of an intron. Transcription of both genes was significantly stimulated by the intron. The introns have a direct role in enhancing transcription of *INO1* and *ASC1* because there was a marked increase in nascent transcripts from these genes in the presence of an intron. Intron-mediated enhancement of transcription required a splicing competent intron. Interestingly, both *INO1* and *ASC1* were in a looped configuration when their genes contained an intron. Intron-dependent gene looping involved a physical interaction of the promoter and the terminator regions. In addition, the promoter region interacted with the 5' splice site and the terminator with the 3' splice site. Intron-mediated enhancement of transcription was completely abolished in the looping defective *sua7-1* strain. No effect on splicing, however, was observed in *sua7-1* strain. On the basis of these results, we propose a role for gene looping in intron-mediated transcriptional activation of genes in yeast.**

chromosome conformation capture | RNA polymerase II

The protein encoding genes in eukaryotes differ from their prokaryotic counterparts in having noncoding intervening regions called introns, which are removed by splicing to generate mature mRNA. Since their discovery in 1977, there has been considerable debate regarding the functional role of introns in eukaryotes (1). It is widely believed that introns increase proteomic complexity by facilitating expression of multiple proteins from a single gene by alternative splicing (2). In budding yeast, where more than 95% of genes are without introns and there are very few instances of alternative splicing, introns do not contribute significantly to the proteomic diversity (3). The presence of introns in all eukaryotes, despite the high cost of maintaining them and the existence of the elaborate splicing machinery needed to remove them, suggest that introns are playing a more fundamental and evolutionarily conserved role in eukaryotic cells.

One role of introns that has been remarkably conserved among diverse organisms, and which confers an additional advantage to eukaryotic genes, is their effect on efficiency of gene expression (4–6). Introns significantly enhance the transcriptional output of genes that harbor them. The expression level of intronless transgenes in mammalian cells is often 10–100 times lower than their intron-containing counterparts (5). The inclusion of just one intron near the 5' end of the gene increases transcription of the gene many folds. A number of mammalian genes, including  $\beta$ -globin, growth hormone, thymidylate synthase, purine nucleoside phosphorylase, cathepsin L, and HIV-1 require introns for their normal expression (7–14). A similar intron-dependent stimulation of transcription has been observed in a variety of genes in plants (6, 15).

In yeast, less than 5% of genes contain introns, but these intron-containing genes produce approximately 27% of total cellular mRNA (4, 16). On average, intronic genes produce 3.9-fold more mRNA than their nonintrinsic counterparts in yeast (3). Removal of introns from yeast genes, like in plants and mammalian systems, decreases their mRNA output (3, 17). Deletion of introns from several yeast genes affected their transcription sufficiently to cause a phenotypic growth defect (3, 18). Introns also have been shown to stimulate transcription in flies. However, there are genes in all classes of organisms whose expression remains high irrespective of the presence or absence of introns, whereas a minority of genes are negatively regulated by introns (5, 6). In some cases, introns increase translational output without any increase in transcription of the gene by either affecting the stability of mRNA or by facilitating transport of mRNA out of the nucleus into the cytoplasm (5, 6).

The intron-mediated stimulation of transcription is physiologically relevant for several reasons: (i) A significant number of eukaryotic genes exhibit the phenomenon; (ii) it is an evolutionary conserved feature that is exhibited by such diverse organisms as yeast, nematodes, flies, plants, and mammals; and (iii) it often confers fitness to the organism by allowing tissue specific expression or developmentally regulated expression.

Intron-mediated transcriptional regulation can be broadly divided into two categories: (i) splicing-independent regulation and (ii) splicing-dependent regulation. Introns can often stimulate transcription because of the presence of an enhancer or a promoter element within their sequence (6). Such introns can influence transcription even if their orientation is reversed (19). In contrast, splicing-dependent regulation of transcription requires a functional, splicing-competent intron within the body of the gene. Such introns cannot affect transcription if their splicing is compromised by a mutation in the conserved sequences at the 5' splice site, 3' splice site, branchpoint or if they are inserted in an antisense orientation (8, 17). This direct effect of introns on transcription of genes is often referred to as “intron-mediated enhancement” (IME) and will be the focus of this investigation (6). IME requires the presence of an intron near the 5' end of the gene. It has been proposed that a promoter proximal 5' splice site facilitates recruitment of the transcription machinery to the promoter and, therefore, helps in initiation of transcription. The interaction of U1 snRNA with general transcription factor TFIIF and the effect of this interaction on reinitiation of transcription provided support to this hypothesis (20). The precise mechanism of intron-dependent enhancement of transcription, however, is not entirely clear.

Here, we show that inclusion of an intron in *INO1* resulted in constitutive activation of the gene. The intron-mediated

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activation was due to an increase in nascent transcription of *INO1*, required splicing, and was independent of the transcription activator Ino2. In the presence of the intron, the promoter of *INO1* interacted with its terminator region to form a gene loop. Similar results were obtained with *ASC1*. Intron-mediated transcriptional activation was abolished in the looping defective *sua7-1* strain. These results suggest a role for gene looping in intron-mediated enhancement of transcription in budding yeast.

## Results

**Intron-Mediated Enhancement of Transcription of *INO1* and *ASC1*.** To investigate intron-dependent enhancement of transcription in budding yeast, we inserted an intron into an intronless gene and deleted the intron from a natural intron-containing gene. Transcription of the genes was then compared under the intron-plus and intron-minus conditions.

*INO1* is an intronless gene whose transcription is regulated by inositol. The gene is repressed in the presence of inositol and is transcriptionally activated upon depletion of inositol from the growth medium (21, 22). To examine the effect of an intron on transcription of *INO1*, a 308-nt-long *ACT1* intron was inserted in the gene 500 bp downstream of the initiator codon (*SI Appendix*, Fig. 1). The inserted intron was splicing competent because it was efficiently removed from the *INO1* transcripts (*SI Appendix*, Fig. 2C, lanes 5 and 6). Transcription of *INO1* was monitored in cells grown in the presence or absence of inositol by RT-PCR. The intron had little effect on the transcript level of *INO1* under inducing conditions (Fig. 1B, lanes 2 and 4 and C). However, there was a 10-fold increase in steady-state level of *INO1* mRNA in the presence of inositol in the medium under intron-plus condition (Fig. 1B, lanes 1 and 3 and C). Thus, the presence of intron caused an increased accumulation of *INO1* transcripts under noninducing conditions.

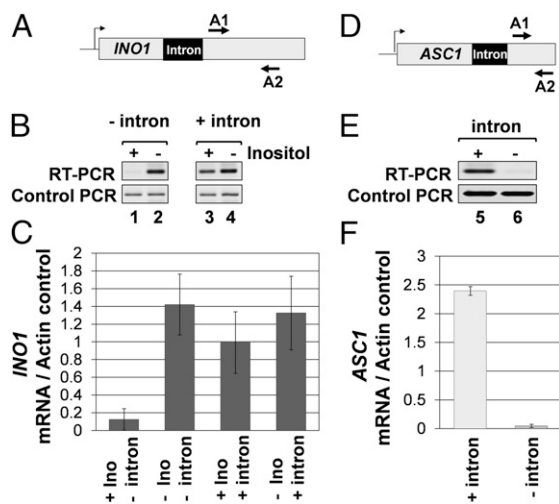
An increase in transcript level of a gene can be attributed either to enhanced transcription of the gene or to an increase in mRNA stability (23). To address this issue, transcription run-on (TRO) analysis of *INO1* was performed in intron-plus and

intron-minus states of the gene. The TRO assay measures the level of nascent transcripts that are still attached to the elongating polymerase on the template. In the native intron-less *INO1*, only a weak TRO signal could be detected on the gene when the cells were grown in inositol-containing medium (*SI Appendix*, Fig. 3B, lanes 1–8 and C). In the presence of an intron, however, there was a marked increase in TRO signal throughout the gene under noninducing conditions (*SI Appendix*, Fig. 3E, lanes 25–29 and F). These results clearly indicate that the intron-dependent increase in RNA level of *INO1* under noninducing conditions is due to transcriptional activation of the gene. Furthermore, the presence of the intron conferred constitutive activation of *INO1* (Fig. 1C and *SI Appendix*, Fig. 3F).

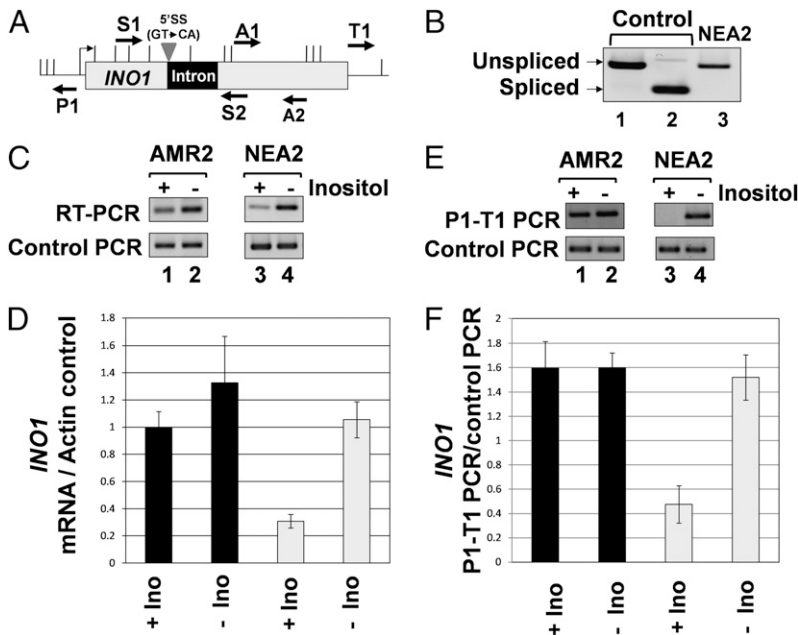
We next asked whether deletion of the native intron from *ASC1* had a reciprocal effect on gene expression. *ASC1* has a single 272-nt-long intron positioned 583 bp downstream of the start codon (Fig. 1D). The intron was precisely removed from the chromosomal copy of the gene (*SI Appendix*, Fig. 4). Deletion of the intron resulted in a 25-fold decrease in the transcript level of *ASC1* (Fig. 1E, lanes 5 and 6 and F). TRO analysis revealed at least a 10-fold decrease in nascent transcription of *ASC1* after removal of the intron (*SI Appendix*, Fig. 5B, lanes 2–4 and 9–11 and C). Similar results were reported earlier with a plasmid borne copy of *ASC1* (17). An increase in TRO signal at the 3' end of *ASC1* was observed in the absence of intron (*SI Appendix*, Fig. 5B, lane 12). This result could be due to the presence of a promoter for transcription of anti-sense RNA at the 3' end of *ASC1*. To check this possibility, we performed RT-PCR analysis for the *ASC1* anti-sense transcript near the 3' end of the gene. We could not detect any signal for anti-sense RNA at the 3' end of intron-less *ASC1* (*SI Appendix*, Fig. 6B, lane 1), but we could observe the signal for sense transcript (*SI Appendix*, Fig. 6B, lane 3). These results suggest that TRO signal at the 3' end of intron-less *ASC1* is not due to anti-sense transcription but due to the accumulation of transcribing RNAP II molecules near the terminator region of *ASC1*. The overall conclusion of these experiments is that both *INO1* and *ASC1* exhibit intron-dependent activation of transcription in yeast cells.

**Intron-Mediated Enhancement Depends on Splicing.** Introns located in the 5' UTR of several plant and mammalian genes function as enhancer elements (6). Such introns bring about transcriptional activation of genes in a splicing-independent manner. To determine whether intron-mediated enhancement of *INO1* transcription was due to the splicing function of intron, the 5' splice site of *ACT1* intron was mutated from GT to CA as described in Furger et al. (17). The mutant intron was inserted in *INO1* gene as described. As expected, the intron with a mutated 5' splice site could not be spliced out of *INO1* precursor mRNA, and a longer transcript was produced (Fig. 2B, lane 3). Results from RT-PCR analysis revealed that the intron-mediated transcriptional stimulation of *INO1* under noninducing conditions decreased by approximately 60–70% in the presence of a splicing defective intron (Fig. 2C, lane 3). Thus, the intron-mediated increase in transcription of *INO1* under noninducing conditions depends on its splicing function.

Activated transcription of *INO1* requires the transcription activator Ino2, which is constitutively bound to the *UAS* element of *INO1* but brings about stimulation of transcription only in the absence of inositol (21, 24). To determine whether intron-mediated enhancement of *INO1* requires Ino2, we repeated RT-PCR in an *ino2<sup>-</sup>* strain containing *INO1* with an intron. The intron-mediated constitutive activation of *INO1* was maintained in *ino2<sup>-</sup>* strain (*SI Appendix*, Fig. 7B, lanes 1 and 2). These data indicate that intron-dependent enhancement of *INO1* transcription does not require the activator protein.



**Fig. 1.** The presence of an intron leads to increased accumulation of *INO1* and *ASC1* mRNA. (A and D) Schematic depiction of intron-containing *INO1* and *ASC1* genes indicating A1 and A2 primers used in RT-PCR analysis. (B and E) RT-PCR analysis of *INO1* and *ASC1* genes with and without an intron. The reverse transcription was performed by using oligo dT, and the cDNA obtained was PCR amplified by A1 and A2 primers. The control PCR represent the *ACT1* RNA levels, which is used as a loading control indicating that equal amounts of template were present in each RT-PCR. (C and F) Quantification of transcript levels of the intron-less and intron-containing *INO1* and *ASC1*.



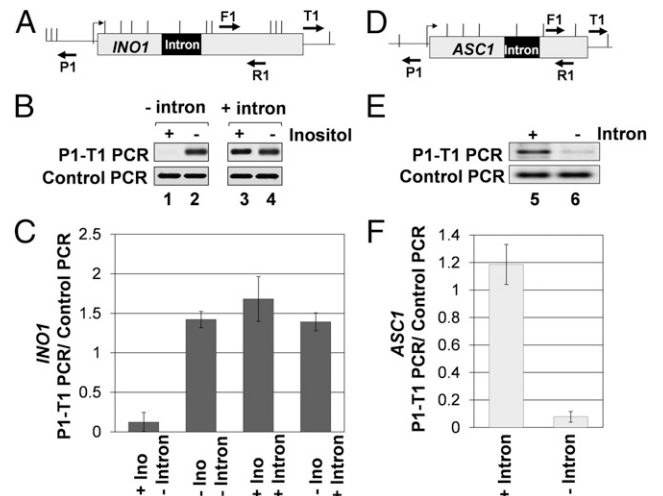
**Fig. 2.** Splicing is required for intron-dependent enhancement of *INO1* transcription and gene looping. (A) Schematic depiction of *INO1* with a 5' splice site mutated intron indicating the position of AluI and EcoRV restriction sites (vertical lines) and PCR primers used in RT-PCR and CCC analysis. The strain with the mutated 5' splice site is NEA2, whereas the strain with the wild-type splice sites is AMR2 (B) *ACT1* intron with a mutated 5' splice site is not removed from *INO1* pre-mRNA as indicated by the position of RT-PCR product obtained by using primers S1 and S2 flanking the intronic region of *INO1* (lane 3). Control PCRs with and without intron were obtained by using genomic DNA from AMR2 and BY4733 as template using primers S1 and S2. (C) RT-PCR analysis of *INO1* harboring a 5' splice site mutated intron. The reverse transcription was performed by using oligo dT, and the cDNA obtained was PCR amplified by using A1 and A2 primers. The control PCR represent the *ACT1* mRNA levels that are used as a loading control, indicating that equal amount of template was present in each of the RT-PCRs. (D) Quantification of the data shown in C. (E) CCC analysis of *INO1* containing a 5' splice site mutated intron. The PCR products are indicated by the primers used for amplification. A P1-T1 PCR product indicates a looped configuration. The A1-A2 PCR represent the loading control, indicating that equal amounts of template DNA were present in each CCC PCR. (F) Quantification of the data shown in E.

**Gene Looping Accompanies Intron-Mediated Enhancement of Transcription.** Gene looping is defined as the physical interaction of the promoter and terminator regions of a gene in a transcription-dependent manner (25). We earlier demonstrated that Ino2-mediated transcriptional activation of *INO1* is accompanied by formation of a looped gene configuration (21). We also showed that gene looping accompanies activator-dependent increase in transcription of other yeast genes as well. We therefore asked whether intron-mediated enhancement of *INO1* transcription also results in the formation of a looped structure. To detect gene loops, the chromosome conformation capture (CCC) assay was used. We have used this assay to show transcription-dependent looping of several yeast genes (21, 25, 26). A PCR product obtained by using divergent primers P1 and T1 was taken as a measure of the interaction of the promoter and terminator regions in these experiments (Fig. 3A and D). To find out whether intron-mediated enhancement is accompanied by gene looping, CCC analysis of intron-containing *INO1* was carried out in the cells grown in the presence and absence of inositol. A robust P1-T1 looping signal was obtained for intronic *INO1* both in the presence and absence of inositol (Fig. 3B, lanes 3 and 4). This result is in contrast to nonintronic *INO1*, which exhibited gene looping only in the absence of inositol (Fig. 3B, lane 2). The extent of gene looping in the presence of the intron was almost the same under inducing and noninducing conditions (Fig. 3B and C). The insertion of intron with the mutated 5' splice site did not result in a looped gene configuration of *INO1* under noninducing conditions (Fig. 2E, lane 3). Similar results were obtained with *ASC1*. In the presence of the intron, CCC analysis of *ASC1* yielded a distinct P1-T1 PCR product (Fig. 3E, lane 5). This signal was reduced more than 10-fold after removal of the intron from the gene (Fig. 3E, lane 6 and F). These analyses reveal that both *INO1* and *ASC1* are in a looped configuration during intron-mediated enhancement of transcription.

CCC mapping of looping interactions across the *INO1* and *ASC1* genes revealed that the promoter, in addition to contacting the terminator (Fig. 4C, lane 14 *Top* and *Middle* and E, lane 24), also interacted with the region near 5' splice site (Fig. 4C, lane 11 *Top* and *Middle* and E, lane 21). Furthermore, the terminator region made contact with the 3' splice site (Fig. 4C, lane 17 *Top* and *Middle* and E, lane 26). We were, however, unable to detect a direct interaction of the 5' splice site with the 3' splice site (Fig.

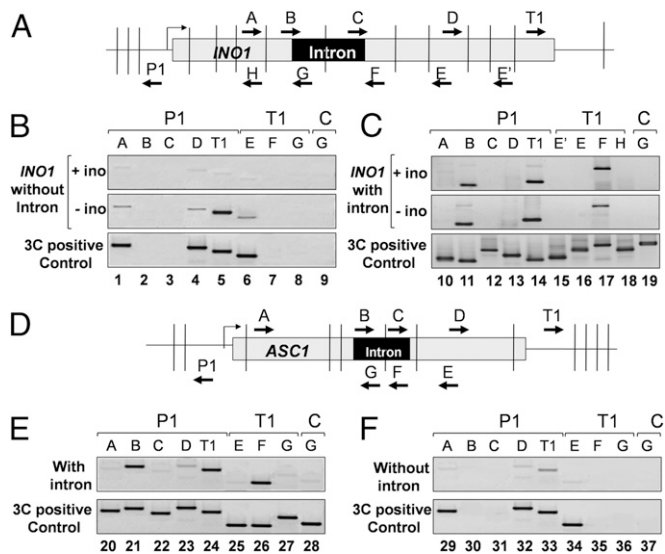
4C, lane 19 *Top* and *Middle* and E, lane 28), and we did not detect interaction of the promoter or terminator with any other internal region of either genes (Fig. 4C, lanes 10, 12, 13, 15, 16, and 18 *Top* and *Middle* and E, lanes 20, 22, 23, 25, and 27). A similar interaction of the intron with the promoter and terminator regions was observed during gene looping of human *BRCA1* (27).

**Gene Looping Has a Possible Role in Intron-Mediated Enhancement of Transcription.** The results described above demonstrate looping of *INO1* and *ASC1* during intron-mediated enhancement of transcription. It was, however, not clear from these experiments whether



**Fig. 3.** Gene looping accompanies intron-mediated stimulation of transcription of *INO1* and *ASC1*. (A and D) Schematic depiction of the intron-containing *INO1* and *ASC1* genes, indicating the position of restriction sites (vertical lines) and PCR primers used in CCC analysis. (B and E) CCC analysis of intronless and intron-containing *INO1* and *ASC1*. The PCR products are indicated by the primers used for amplification. A P1-T1 PCR product indicates a looped configuration. The F1-R1 PCR represent the loading control indicating that equal amounts of template DNA were present in each CCC PCR. (C and F) Quantification of the CCC results shown in B and E.





**Fig. 4.** Interaction of splice sites with the promoter and terminator regions during gene looping. (A and D) Schematic depiction of intron-containing *INO1* and *ASC1*, indicating the position of restriction sites (vertical lines) and PCR primers used in the high-resolution CCC mapping. (B, C, E, and F) PCR products derived from the indicated primer pairs by CCC analysis show that the promoter interacts with the 5' splice site and the terminator with the 3' splice site in the intron-containing *INO1* and *ASC1*. The CCC positive control PCR products were generated to check the amplification efficiency of the primer pairs in the PCR.

gene looping was responsible for intron-mediated stimulation of transcription. To address the issue, we analyzed transcription of intron-containing *INO1* in the looping-defective TFIIB mutant, *sua7-1* (28). TFIIB is an important determinant of gene looping in budding yeast (21, 29). The *sua7-1* mutation abolishes gene looping (29). This mutant provides a convenient way to determine whether a cellular process depends on gene looping. Using this mutant, it was demonstrated that gene looping is required for maintenance of “transcriptional memory” in yeast (30, 31). Thus, to determine whether gene looping is required for intron-mediated enhancement, we assayed transcription of *INO1* with an intron in *sua7-1* cells by the RT-PCR approach. We did not observe any intron-mediated increase in the RNA level of *INO1* under noninducing conditions in *sua7-1* cells (Fig. 5 B, lane 5 and C). In a parallel experiment, CCC analysis showed the loss of gene looping of intronic *INO1* in *sua7-1* cells (Fig. 5 D, lanes 11 and 12 and E). These results demonstrate that intron-mediated constitutive enhancement of *INO1* transcription was abolished in the looping defective *sua7-1* mutant. Because splicing is required for intron-mediated stimulation of *INO1* (Fig. 2), gene looping may directly influence intron-dependent enhancement of transcription or it may do so indirectly by affecting splicing. To clarify the issue, we checked for a possible splicing defect in *sua7-1* strain by RT-PCR (SI Appendix, Fig. 2D). The primers were designed so that a spliced RNA will give a shorter PCR product, whereas an unspliced RNA will give a longer PCR product because of the presence of intron (SI Appendix, Fig. 2A). We found that the intron-containing *INO1* precursor mRNAs were spliced as efficiently in *sua7-1* cells as in wild-type cells (SI Appendix, Fig. 2 C, lanes 5 and 6 and D, lane 9). This observation ruled out the possibility of gene looping indirectly influencing intron-dependent transcription through splicing. These results suggested a more direct role for gene looping in intron-mediated enhancement of transcription. The possibility of the TFIIB mutation (*sua7-1*) affecting intron-mediated transcriptional activation

through an aspect of transcription other than gene looping, however, still could not be ruled out.

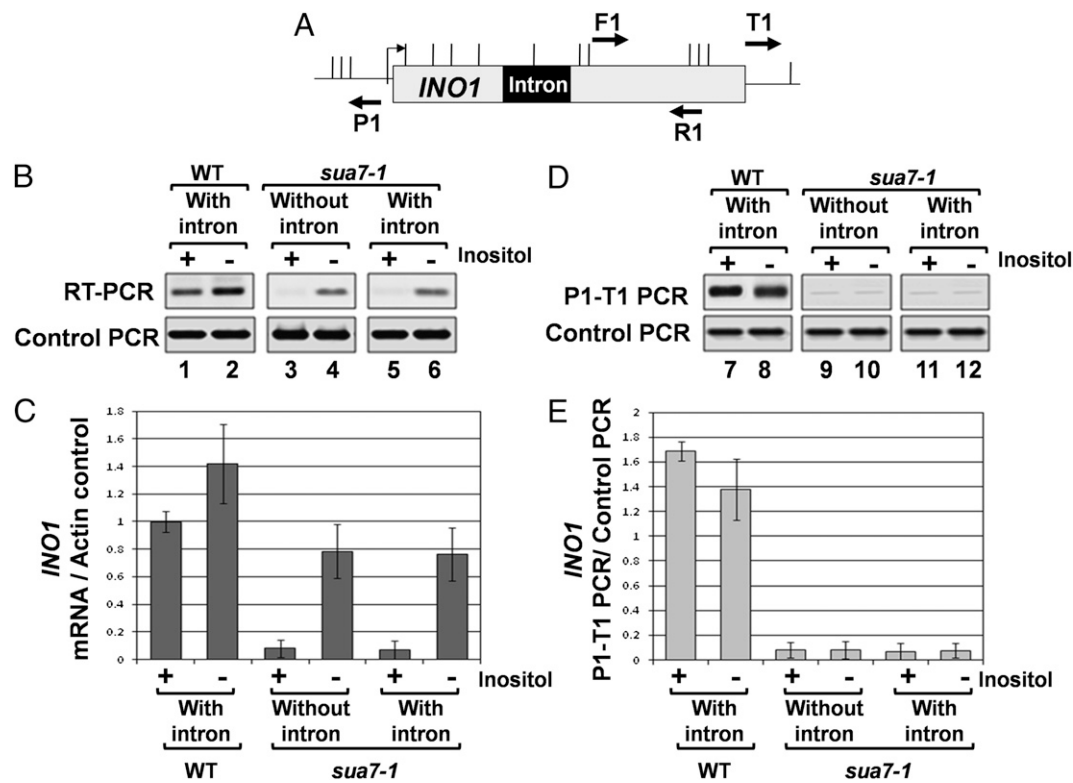
Because gene looping of intronic *INO1* involved juxtaposition of the promoter and terminator regions with each other, and with the ends of the intron, we next examined the interaction of 5' and 3' splice site with the promoter and terminator regions, respectively, in *sua7-1* strain. We found that not only was the promoter-terminator contact abolished in the looping defective strain, but interaction of promoter with the 5' splice site and the terminator with the 3' splice site was also abrogated in *sua7-1* cells (SI Appendix, Fig. 8B). Thus, there was a complete loss of intron-dependent looped gene architecture in looping defective cells.

## Discussion

Here, we provide evidence that intron-mediated enhancement of transcription in yeast requires a splicing-competent intron and occurs when the gene is in a looped configuration. The presence of an intron may facilitate juxtaposition of the promoter and the terminator regions of a gene resulting in a looped gene structure. In the looping-defective *sua7-1* mutant, although splicing was normal, there was no enhancement of transcription. The intron-mediated enhancement of transcription is therefore not due to splicing per se but may be due to the formation of a splicing-dependent looped architecture of the gene.

It was demonstrated that a 5' splice site alone can bring about an increase in transcription of HIV-1 and  $\beta$ -globin genes (10). The enhancement of transcription elicited by a 5' splice site, however, was much lower (75% less) compared with that brought about by a full-length intron. Our results show that in a looping defective *sua7-1* strain, even a splicing competent intron cannot evoke a transcription activation response. The results presented here suggest that it is not merely the presence of a 5' splice site or an intron, but an intron-facilitated looped gene configuration, that confers transcriptional enhancement of a gene. We further show that during intron-dependent gene looping, there are additional contacts between the promoter and 5' splice site and the terminator and 3' splice site (Fig. 6). The promoter–5' splice site interaction explains why the 5' splice site alone is able to bring about a modest increase in transcription of a gene on its own. However, to achieve the intron-mediated enhancement to the fullest possible extent, further contacts of the promoter region with the terminator are essential. How the presence of an intron facilitates gene loop formation is not yet clear. We propose that it is the interaction of 5' splice site with the promoter and 3' splice site with terminator that bring the two ends of a gene in close physical proximity and facilitate the promoter-terminator contact.

Because looping-defective *sua7-1* strain has a mutated TFIIB, it is possible that the loss of intron-mediated enhancement of transcription in *sua7-1* mutant is not due to impairment of gene loop formation, but due to some other transcription defect associated with this mutation. The *sua7-1* codes for a mutated form of TFIIB with glutamic acid at position 62 being replaced by lysine (TFIIB-E62K). The mutant is cold sensitive and exhibits altered transcription start site selection (32). The activator-dependent transcription that requires gene looping exhibited a kinetic lag in the *sua7-1* mutant (21, 30). The binding affinity of TFIIB-E62K for the promoter region and its interactions with TBP and RNAP II are comparable to that of wild-type TFIIB (33). The recruitment of general transcription factors and RNAP II onto the promoter during assembly of preinitiation complex is also normal in the presence of TFIIB-E62K (33). The cross-linking of mutated TFIIB to the terminator region of actively transcribed genes, however, is severely compromised in *sua7-1* cells (29). The localization of wild-type TFIIB to the 3' end of genes is also abolished in the looping-defective mutants of Ssu72 and Rna15, which are 3' end processing/termination factors (21,



**Fig. 5.** Intron-dependent enhancement of transcription and gene looping are abolished in *sua7-1* strain. (A) Schematic depiction of intron-containing *INO1*, indicating the position of AluI and EcoRV restriction sites (vertical lines) and PCR primers used in CCC analysis. (B) RT-PCR analysis of intronless and intron-containing *INO1* in wild-type and *sua7-1* strains. The reverse transcription was performed by using oligo dT, and the cDNA obtained was then PCR amplified by A1 and A2 primers shown in Fig. 1. The control PCR represents the *ACT1* mRNA levels, which are used as a loading control, indicating that equal amounts of template was present in each of the RT-PCRs. (C) Quantification of RT-PCR results shown above in B. (D) CCC analysis of intronless and intron-containing *INO1* in wild-type and *sua7-1* strains after 3 h of induction. The PCR products are indicated by the primers used for amplification. A P1-T1 PCR product indicates a looped configuration. The F1-R1 PCR represents the loading control, indicating that equal amounts of template DNA were present in each CCC PCRs. (E) Quantification of the CCC results shown above in D.

25, 29). Because TFIIB has been shown to physically interact with Ssu72 and Rna15 (21, 34, 35), it has been proposed that TFIIB interaction with the terminator-bound factors is the molecular basis of gene looping (21, 25, 29). Accordingly, we recently purified a complex of TFIIB with a number of terminator-bound factors from yeast cells (36). This TFIIB-termination factor complex, which has been proposed to facilitate gene loop formation by bridging the promoter and the terminator regions, was not observed in looping-defective *sua7-1* cells. The overall conclusion of these results is that TFIIB-E62K (*sua7-1*) is defective in its interaction with the terminator-bound factors and, consequently, transcriptionally activated genes are no longer in a looped configuration. Taken together, these results suggest a role for gene looping in intron-mediated enhancement of transcription in yeast cells.

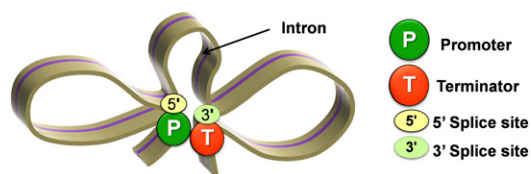
A critical issue is how intron-dependent gene looping brings about enhancement of transcription. It has been proposed that when a gene is in a looped configuration, the proximity of the

terminator to the promoter region facilitates release of polymerase from the terminator region. The polymerase is then recycled back to the juxtaposed promoter for reinitiation of transcription. Such a coupling of termination to reinitiation, with a concomitant increase in the transcriptional activity, has been demonstrated for RNAP III, RNAP I, mitochondrial polymerase, and archaeal polymerase (37–41). Recently, the terminator-promoter cross-talk also was shown during transcription by RNAP II (42). Several possible mechanisms have been proposed to explain transfer of polymerase from the terminator to the promoter for reinitiation (43). Gene looping is by far the most attractive of these proposed mechanisms. However, rigorous experimental evidence is needed to show that gene looping facilitates the release and transfer of RNAP II from the 3' end to the 5' end of a gene after termination of transcription. Nevertheless, this study provides an insight into the mechanism of intron-mediated enhancement of transcription in yeast that also may help understand the phenomenon in higher eukaryotes.

### Materials and Methods

**Yeast Strains.** The yeast strains used in this study are listed in *SI Appendix, Table S1*. Strains BY4733, AMR2, AMR6, and AMR15 are isogenic. Strain AMR2 was derived from BY4733 by introducing an intron in the *INO1* gene at the 500 bp position of the ORF as described in Cheng et al. (44). Strain AMR6 was derived from AMR2 by inserting the kanamycin resistance gene (*kanMX6*) between *INO1* and the downstream *SNA3* genes. Strain AMR15 was derived from AMR2 by replacing the entire ORF of *INO1* by *KanMX6* as described in Wach et al. (45).

AMR14 was derived from W303-1A by first replacing the entire ORF of *ASC1* by *KanMX6* as described in Wach et al. (45). Second, an intron-less



**Fig. 6.** A model of intron-dependent gene looping.

*ASC1* was obtained by PCR amplifying *ASC1* cDNA using primers that contained adaptor sequences homologous to the regions upstream and downstream of *ASC1* ORF. Third, the intron-less *ASC1* was inserted back into the yeast genome by homologous recombination. Replica plating was performed to make sure that the *KanMX6* marker was lost, and the correct insertion of the intronless *ASC1* gene was verified by PCR.

Strain MHA1 was derived from YMH124 by introducing an intron in the *INO1* gene at the 500 bp position of the ORF as described earlier. NEA2 is identical to AMR2 except that the 5' splice site of the intron integrated into *INO1* was mutated from GT to CA.

**Cell Culture.** Cells for RT-PCR and CCC analyses of *INO1* were grown as described in El Kaderi et al. (21). Induction of *INO1* in all experiments was performed by growing cells for 3 h in inositol-depleted medium. For *ASC1*, cells were grown in YP-dextrose till  $A_{600}$  reached 0.4. Cells were then harvested and processed for RT-PCR or CCC.

**RT-PCR.** Isolation of RNA and RT-PCR analyses of *INO1* and *ASC1* was performed as described in El Kaderi et al. (21).

**CCC.** CCC analyses for *INO1* and *ASC1* was performed essentially as described in El Kaderi et al. (21), except that chromatin was solubilized with 1% SDS before restriction digestion. For CCC analysis of *INO1*, chromatin was

digested with AluI and EcoRV, whereas restriction digestion was performed with AluI, DraI, and NlaIV for CCC analysis of *ASC1*. Position of P1, T1 and other primers, and F1 and R1 control primers is indicated in figures. The CCC-positive control PCR products in Fig. 4 were generated as described in El Kaderi et al. (21).

**TRO.** TRO assay was performed by the modification of protocols described in Birse et al. and Hirayoshi and Lis (46, 47). All TRO signals were quantified by using GEL LOGIC 200 (Kodak) system and normalized with respect to *ACT1* control.

**Quantification.** All quantifications and data analyses were performed as described in El Kaderi et al. (21).

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