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# The enhancement of ribosomal transcription by the recycling of RNA polymerase I

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## SUMMARY

It has been suggested that the tandemly repeated ribosomal genes of eukaryotes may be subject to a special mechanism of transcriptional enhancement, called Readthrough Enhancement, in which transcription factors are recycled. Recent experiments with the mouse ribosomal genes, although consistent with this possibility, were unable to distinguish between true Readthrough Enhancement and promoter occlusion. To test directly for Readthrough Enhancement, the pre-ribosomal RNA of *Xenopus laevis* was prematurely terminated within the 18S gene on a circular template. This premature termination was found to reduce the efficiency of pre-ribosomal RNA promotion *in cis* by 80 to 90%. Since the pre-ribosomal RNA is normally terminated only 213 base pairs upstream of its own initiation site, the results strongly suggest that the recycling of RNA polymerase, or Readthrough Enhancement, is a means by which ribosomal transcription is enhanced in *Xenopus laevis*.

## INTRODUCTION

Ribosomal transcription in *Xenopus laevis* is enhanced by sequences which lie within the ribosomal DNA spacer, (rDNA spacer), (1). Two distinct sequence elements have been shown to be involved in this enhancement, the 60/81 base pair (b.p.) repetitive "enhancer" elements (2,3) and the Spacer Promoters (4), see fig. 1. The 60/81 b.p. elements probably bind RNA polymerase I or some other transcription factor. The Spacer Promoters are themselves unable to transcribe the ribosomal genes, since their transcripts are terminated just upstream of the pre-ribosomal RNA (pre-rRNA) promoter, (1), site "T" in fig. 1. However they indirectly increase the rate of pre-rRNA transcription by amplifying the enhancement effect of the 60/81 b.p. repetitive elements lying downstream of them, (5) for a review see ref. 6.

Contrary to earlier ideas, transcription of the *X. laevis*

ribosomal genes does not terminate after completion the 40S pre-ribosomal RNA, (40S pre-rRNA), but continues through the sequences lying downstream of the 28S gene, (7,8). The transcription is eventually terminated just 213 b.p. upstream of the pre-rRNA promoter for the following gene unit in the tandem gene array, site "T" in fig. 1, i.e. the same site at which the Spacer Promoter transcripts are terminated. Thus the greater part of the region previously refered to as the non-transcribed spacer (NTS), is actually a 3'-transcribed spacer (3'-ETS), see fig. 1. The true NTS is only 212 b.p. in length and 145 b.p. of this is occupied by the pre-rRNA promoter. Hence polymerase molecules actually transcribe to within about 4 polymerase diameters of the downstream pre-rRNA promoter. It is therefore possible that these polymerase molecules and/or their associated factors, on completing transcription of one cistron, are captured by the downstream cistron and re-utilised. Pre-rRNA initiation would then no longer depend solely on random collisions between the promoter or the spacer enhancer elements and the polymerase. Thus, yet a further mechanism to enhance the rate of ribosomal transcription may occur.

Recycling of transcription factors could function either through release of the polymerase from the template and its recapture by the neighbouring promoter, or by a mechanism in which transcription is terminated but the polymerase is not released. In either case, the local polymerase concentration at the downstream pre-rRNA promoter would be higher than in the free pool. This hypothetical mechanism of transcription enhancement was suggested some years ago (1) and has been called "Readthrough Enhancement" (5).

Our present model of the ribosomal spacer has the Spacer Promoters involved in a stage of gene activation, while the 60/81 b.p. elements directly bind polymerase and/or other transcription factors. Possibly transcription from the Spacer Promoters presents the downstream 60/81 b.p. elements to best advantage, by placing them in an "open" chromatin structure. Once activated, the pre-rRNA promoter may then assume this role and the Spacer Promoters could be repressed without effecting pre-rRNA initiation (5). This might begin to explain why the activity of

these promoters is not always detected (6). Transcription of the 3'-ETS may have the further effect of sweeping transcription factors bound by the 60/81 b.p. elements, towards the pre-rRNA promoter, (1). Readthrough Enhancement would further increase the efficiency with which transcription factors were captured, by simply increasing the time for which each factor is engaged with the template. Transcription complexes initiated at the Spacer Promoters could also be a source of factors for Readthrough Enhancement. However, we believe that when rRNA is being synthesised at a steady rate, transcription from the Spacer Promoters may not be sufficient to be of importance in Readthrough Enhancement, (1,5).

McStay and Reeder, (9), have investigated the role of the -213 b.p. termination site in the promotion of ribosomal transcription in *X. laevis*. By introducing mutations within the terminator, or by changing the spacing between terminator and promoter, these authors showed that the rate of pre-rRNA initiation *in cis* could be greatly reduced in *in vitro* assays. However, this effect was shown to be independent of termination itself, since it occured on both linear and circular templates. Thus McStay and Reeder concluded that the -213 b.p. termination site acted as an upstream element of the pre-rRNA promoter and found no evidence of its involvement in Readthrough Enhancement. Grummt et al. (10) and Henderson and Sollner-Webb (11), have recently identified a termination sequence at about 168 b.p., upstream of the pre-rRNA initiation site of the mouse rDNA. Both sets of authors find that deletion of this termination site reduces pre-rRNA initation at the adjacent promoter. However, in contrast to the results of McStay and Reeder, (9), these effects were only seen when polymerase molecules from upstream initiation sites, transcribed through the pre-rRNA promoter. Grummt et al. (10) and Henderson and Sollner-Webb (11) interpreted these experiments in terms of Readthrough Enhancement. More careful consideration however, suggests that they could not provide a direct demonstration of this phenomenum, since at least some degree of transcriptional interference must have also occured.

Consider the situation of a hypothetical circular rDNA template on which readthrough enhancement occurs. The pre-rRNA

promoter produces a transcript which reads around the template and is terminated just upstream of this same promoter. If the unique termination site were to be inactivated, two changes in transcription would ensue:-

a) Readthrough enhancement would no longer be possible, but also

b) Transcription complexes, initiated at the pre-rRNA promoter, would circulate indefinitely on the template.

The latter could clearly lead to occlusion of the pre-rRNA promoter and hence to transcriptional interference. Promoter occlusion has been shown to occur both in eukaryotes and prokaryotes, even on genes less actively transcribed than the rDNA, (12 to 15).

In an attempt to test directly for Readthrough Enhancement on the *X. laevis* rDNA, we have taken an approach which avoids the problem of promoter occlusion. We argued, that a single ribosomal gene unit placed on a plasmid, may closely ressemble a gene in the *in vivo* tandem array. On the plasmid, the mechanism of Readthrough Enhancement might still function, but now by positive feedback onto the unique pre-rRNA promoter. Premature termination of the pre-rRNA transcript should destroy such feedback and hence eliminate Readthrough Enhancement. The results of this study strongly suggest that Readthrough Enhancement does indeed occur on the *X. laevis* ribosomal genes.

## MATERIALS AND METHODS

**Plasmids.** The plasmid pXl108c (16) contains an EcoRI rDNA fragment from *X. laevis*, inserted into the EcoRI site of pBR-RIII. PBR-RIII is an EcoRI-BamHI deletion mutant of pBR322. In this and subsequent rDNA constructs the suffix -c or -d refer to the two possible orientations of the vector relative to the rDNA. The AluI-BglI fragment from pXl108c, which contains the spacer terminator, (T in fig. 1), was subcloned into the SmaI site of pUC9, after trimming the BglI terminus with $T_4$ DNA polymerase, producing pT1, fig. 2B. The terminator fragment was then recovered from pT1 by EcoRI and AluI digestion and inserted into the AsuII site of pXl108c or pXl$\Delta$ES1c, after filling out the overhanging ends with the klenow fragment of DNA polymerase I,

fig. 2B. Following cloning in *E. coli* HB101, the plasmids were initially selected by restriction analysis, but finally the orientation of the inserted fragment was determined by sequence analysis (17) from an XmnI site, 35 b.p. upstream of the site of insertion. Dimer gene plasmids were constructed by inserting the EcoRI rDNA fragment from pXl108c or from pXlΔNS1c, (fig. 1), into pXlΔES1c or -d, (the respective vector orientations), which had been linearised by partial EcoRI digestion. After cloning in *E. coli* HB101, the appropriate constructs, (figs 4B and 5B), were identified by restriction analysis.

Restriction and DNA modifying enzymes were obtained from Anglian Biotechnology, Boehringer Mannheim, New England Biolabs and P-L Biochemicals.

**Microinjection, and transcript detection.** Plasmids were isolated (18), purified on CsCl-ethidium bromide isopycnic gradients and finally by gel filtration on Sephacel S200. DNA concentrations were determined by UV absorption at 260nm and checked by gel electophoresis and densitometer analyses. Microinjection was carried out as previously described, (1,16), but briefly, *X. borealis* oocytes were injected with 20 nl of plasmid in injection buffer, (88 mM NaCl, 10 mM Tris-HCl, pH 7.4), containing 250 ugm/ml of *a*-amanitin (Boehringer Mannheim). Each oocyte received the molar equivalent of 250 pgm of the 6.6 kb.p. wild type rDNA insert from pXl108c. 30 to 50 oocytes were injected with each plasmid or plasmid mixture and total oocyte RNA was harvested 14 to 24 hrs following microinjection. Total oocyte RNA was extracted as previously described (16).

The pre-rRNA transcripts and readthrough of the ΔNS1 chimearic promoter were detected by S1-mapping (19), using a PstI-TaqI fragment of pXl108c, "pre-rRNA probe" in fig. 1. Transcripts passing through the vector region of the plasmids were detected using the NciI-(EcoRI)-NciI fragment from pBR-RIII, "pBR. probe" in figs 2B and 5B. The probes were $^{32}$P-end labelled, either using $T_4$ polynucleotide kinase, (20) or the klenow fragment of DNA polymerase I. The specific activity of each probe is given in the respective figure legend. Each S1-mapping contained between 10,000 and 50,000 c.p.m. of end-labelled probe, giving a minimum of a 100 fold molar excess

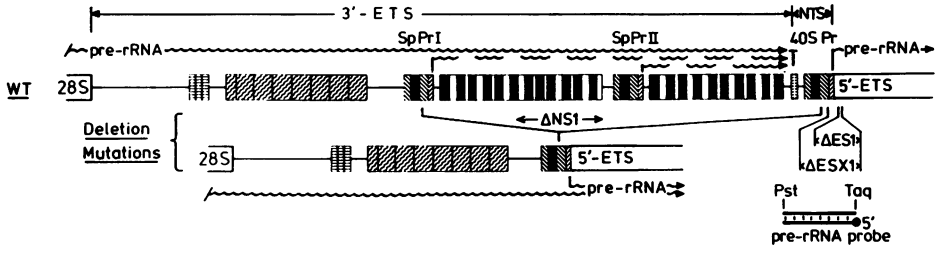


**Figure 1.** The structure of the rDNA inserts of the wild type plasmid, pX1108c, (WT), and the deletion mutant, pX1ΔNS1c, (ΔNS1), as well as the positions of the deletion mutations in pX1ΔES1c, (ΔES1) and pX1ΔESX1c, (ΔESX1). Shading indicates sequence homologies and the known functional sequences, such as the spacer termination site "T", the spacer promoters "SpPr I" and "SpPrII" and the pre-rRNA promoter "40S Pr". "28S" refers to the 3' segment of the 28S gene, "3'-ETS" and "5'-ETS" refer respectively to the External Transcribed Spacers downstream and upstream of the ribosomal genes and "NTS" refers to the short Non-Transcribed (untranscribed) Spacer. The expected pre-rRNA transcripts are indicated by arrows and wavy lines and those from the spacer promoters are dashed. The position and labelling (o-5') of the PstI (Pst) to TaqI (Taq) fragment used as probe for pre-rRNA initiation, is shown.

over the RNAs detected. S1-mapping was carried out, essentially as described (19), in 80% formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.7. The hybridisation temperatures for the pre-rRNA and pBR. probes were 57 and 59°C respectively. Where both probes were used, 59°C was chosen. S1 digestion was carried out with 25 units of enzyme (P-L, Pharmacia) at 45°C in 30 mM Na acetate, 2 mM $ZnSO_4$, 0.2 M NaCl, (pH 4.5) for 30 min. to 1 hr. Protected fragments were analysed on 8% polyacrylamide gels in 8 M urea, 90 mM tris-borate, 2 mM EDTA and detected by autoradiography at -80°C using an intensifying screen (DuPont).

## RESULTS

**Premature Termination of the pre-rRNA transcript.** In order to prematurely terminate the pre-rRNA transcript, the spacer terminator (T in fig. 1) was inserted into the 18S gene region of the wild type rDNA plasmid pX1108c and of the mutant, pX1ΔES1c, see fig. 2B and "Materials and Methods". The ΔES1 mutation, fig. 1, has been used extensively as an internal control, (1,16). It has the characteristics of the wild type pX1108c, but produces a slightly shorter pre-rRNA, which can therefore be distinguished from the wild type pre-rRNA. The pre-rRNA signal from pX1ΔES1c

appears as a double band after S1-mapping, due to the formation of an oligo rA-dT hybrid, which is partially attacked by the S1 nuclease, e.g. see fig. 2A. This has however proved a very reliable way to control the extent of S1 digestion.

Three terminator insertion mutants were chosen for further investigation; one with a single extra terminator inserted in pXl108c in the same orientation as the wild type terminator, (pXlIG1c), a second with two terminators inserted tandemly in this orientation, (pXlIG2c) and a third with the terminator inserted into pXlΔES1c in the corresponding position but the opposite orientation, (pXlIG11c). Each construct was microinjected into *X. borealis* oocytes and the transcripts produced assayed for pre-rRNA initiation, using the 5'-end-labelled PstI -TaqI probe, (fig. 1, pre-rRNA probe) and for pre-rRNA transcripts reading through the vector, using an NciI - NciI probe from pBR-RIII, (fig. 2B, pBR. probe). Comparison in fig. 2A of tracks 1 and 2 with track 3 shows that insertion of the terminator in the wild type orientation very effectively prevented the pre-rRNA transcript from reading through the vector and hence back into the rDNA insert, (Plasmid Transcript). Comparison of track 3 with track 4, shows that the inverted terminator had no effect on this readthrough. Thus the inserted terminator worked in an orientation dependent manner to terminate the pre-rRNA transcript, even when it was transferred to a position within the gene.

**Transcription of the 3'-ETS is necessary for maximal, pre-rRNA initiation.** To test if premature pre-rRNA termination affects the rate of pre-rRNA initiation, pXlIG1c and -2c were co-microinjected with pXlΔES1c, as internal control, fig. 3A, tracks 3 and 4. The results were compared with parallel co-injections of pXl108c with pXlΔES1c and pXl108c with pXlIG11c, the last of these plasmids having the inverted terminator, fig. 3A, tracks 1 and 2. The inserted terminator clearly causes a very significant reduction in pre-rRNA initiation *in cis*, only when placed in the wild type orientation. From the densitometric analyses shown in fig. 3A and three other independent analyses, the efficiency of pre-rRNA initiation on pXlIG1c and -2c was

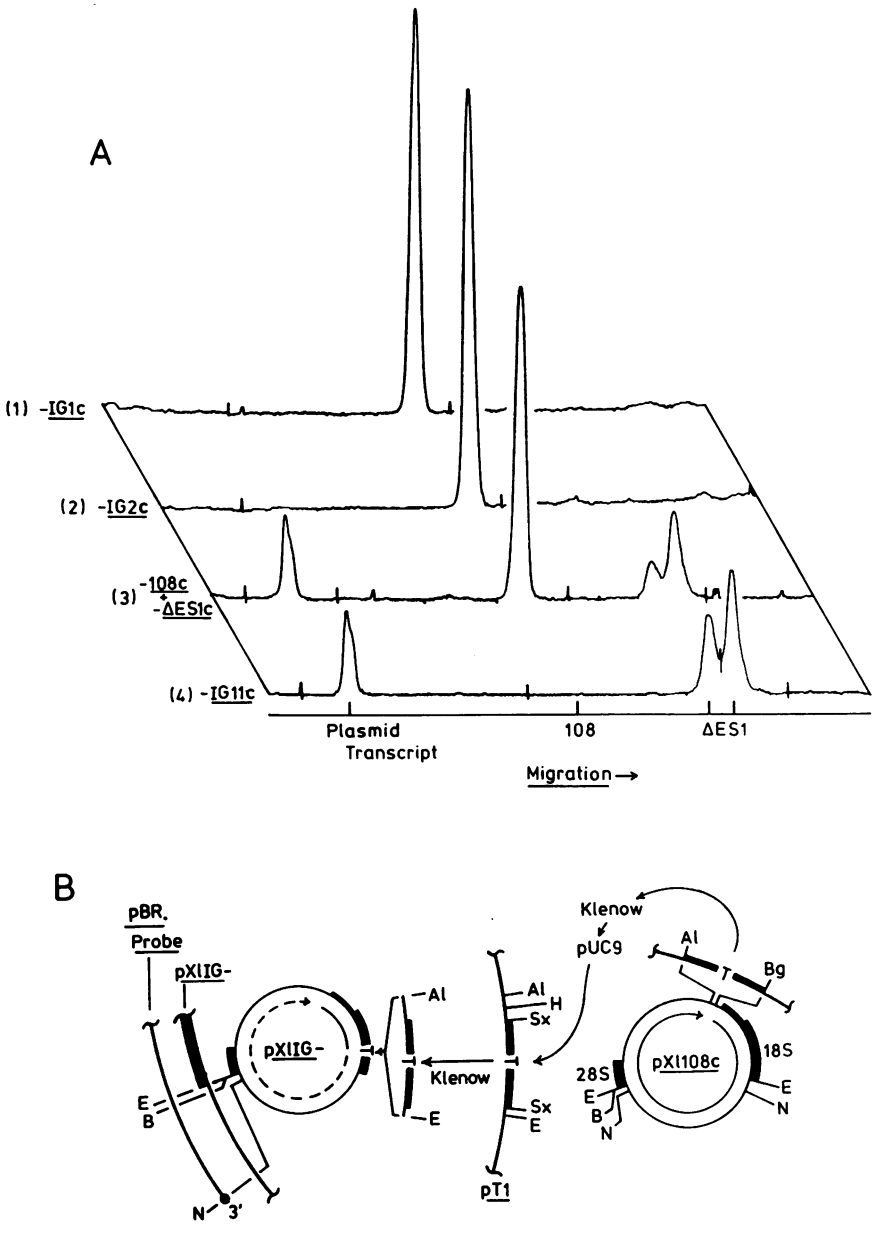


**Figure 2.** Premature termination of the pre-rRNA. The results are shown as densitometer analyses of autoradiographs from a single S1-mapping gel. Tracks 1 to 4 respectively are pXlIG1c (one terminator), pXlIG2c (two terminators), an equimolar mixture of pXl108c and pXlΔES1c (wild type control) and pXlIG11c (reversed terminator). Each track shows a co-analysis with the pBR.,

(0.5x10$^6$cpm/pmole), and pre-RNA, (~0.5x10$^6$cpm/pmole), probes, see (B) below and fig. 1. "108" and "ΔES1" refer respectively to the wild type and control pre-rRNA signals and "Plasmid Transcript" refers to protection of the NciI-(BamHI)-EcoRI region of the pBR. probe. (B) The construction of the terminator insertion mutants used in (A) is shown along with an expansion of the regions probed for transcription of the vector. The 5'-ETS, 18S and 28S gene segments are indicated by thickened lines. Arrows inside the plasmid representations show the expected pre-rRNA transcripts. The S1-mapping probe derived from the standard vector, (pBR-RIII, an EcoRI to BamHI deletion mutant of pBR322) is shown ("pBR. probe") and its labelling indicated (o-5' or o-3'). "B", "E" and "N" are restriction sites for the enzymes BamHI, EcoRI and NciI respectively. The extra spacer terminator, ("T"), was inserted into the 18S gene to produce the pX1IG- series of mutants, in the following manner. The AluI (Al) to BglI (Bg) fragment containing the terminator was inserted into the SmaI (Sx) site of pUC9, destroying the terminal Sma, Alu and Bgl sites and creating pT1. The fragment was then removed from pT1, using flanking AluI (Al) and EcoRI (E) sites and inserted into the AsuII site of the 18S gene of pX1108c or pX1ΔES1c. See also "Materials and Methods".

found to be indistinguishable and only 15 +/-5% of that from the wild type pX1108c.

It was possible that insertion of the terminator had destabilised the pre-rRNA. Hence the above measurements may not have reflected the rates of pre-rRNA initiation but simply the relative steady state RNA levels. To check this possibility, each construct was separately microinjected and pre-rRNA transcription analysed as a function of time. Neither the wild type nor the mutant pre-rRNA molecules showed significant turnover during the first 18 hrs after microinjection, fig. 3B.

Clearly then, premature termination of the pre-rRNA transcript significantly effects the efficiency with which its transcription is promoted. This is exactly the expected effect if readthrough enhancement occured on the microinjected template. However several further criteria can also be tested. Firstly if Readthrough Enhancement occurs, the above effect must be the direct result of prematurely terminating only the pre-rRNA transcript. Secondly, in the absence of premature termination, the majority of pre-rRNA transcription complexes must complete transcription of the template and terminate at the -213 b.p. promoter proximal site.

**Transcription of the microinjected rDNA template is directed solely from the pre-rRNA promoter.** Microinjection of plasmid templates into *Xenopus* oocytes usually leads to significant

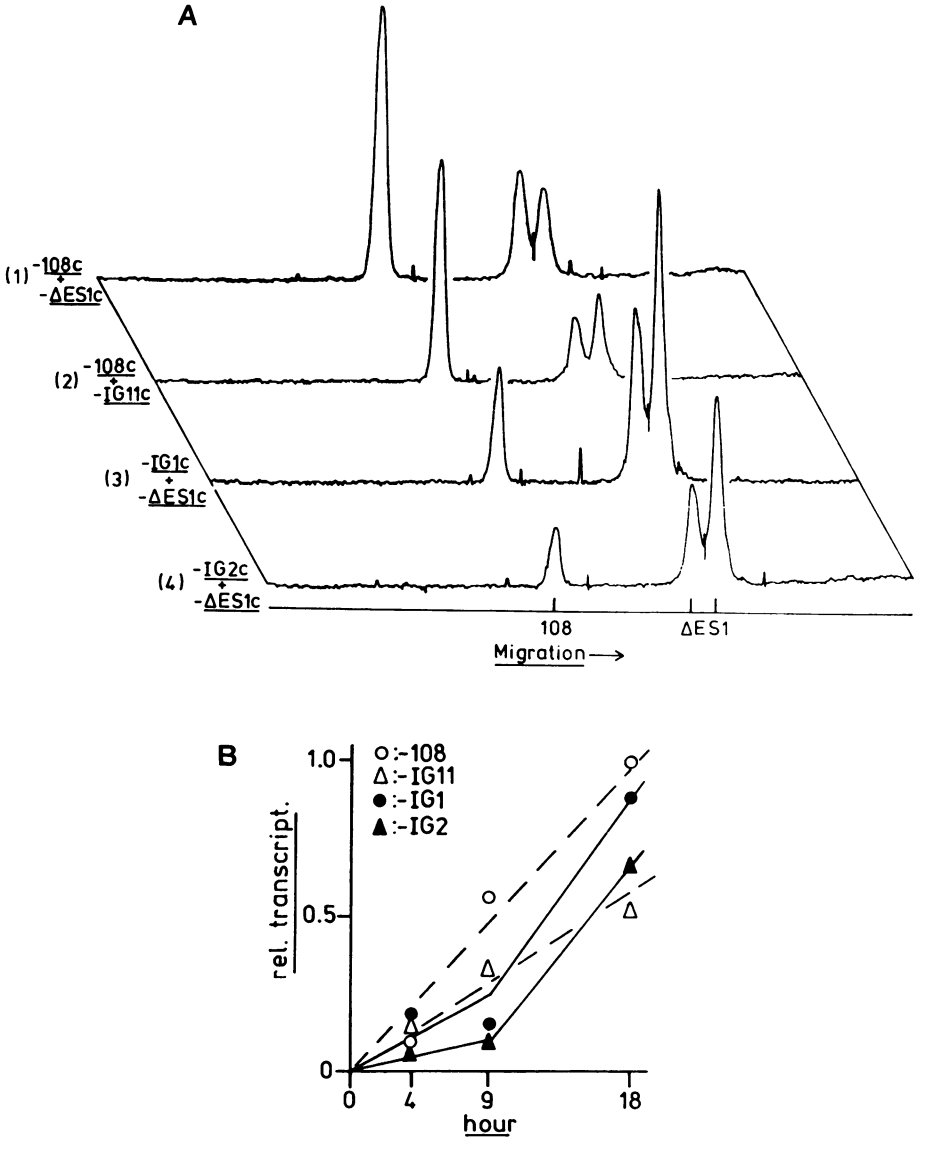


**Figure 3.** (A) Co-injection of terminator insertion mutants with an equimolar amount of a wild type control. The results are shown as a densitometer analysis of a single S1-mapping gel. Tracks 1 to 4 are respectively pX1108c with pX1ΔES1c (wild type), pX1108c with pX1IG11c (reversed terminator), pX1IG1c with pX1ΔES1c (one terminator) and pX1IG2c with pX1ΔES1c (two terminators). Each track shows an analysis with the pre-rRNA probe, (~0.5x10$^6$cpm/pmole), (fig. 1). (B) Stability of the prematurely terminated pre-rRNA transcripts. Each plasmid was separately microinjected, batches of

about 20 oocytes were harvested at the times indicated and these were analysed with the pre-rRNA probe. The pre-rRNA signals were determined by densitometry and plotted after normalisation to the pX1108c, 18 hour signal. 108, IG11, IG1 and IG2 refer to the respective plasmids analysed in (A) above.

levels of non-specific transcription by RNA polymerase II, e.g. see ref. 21. Such transcription, as well as >70% of the RNA polymerase III transcription, was routinely eliminated in our experiments by the co-injection of *a*-amanitin, (16). However it was still possible that RNA polymerases I & III produced significant numbers of "non-specific" transcripts. If this were so, interpreting the effects of premature termination in terms of Readthrough Enhancement would be difficult if not impossible. Thus it was necessary to demonstrate that all transcripts of the coding strand of the microinjected rDNA plasmids, resulted from initiation events at the known pre-rRNA promoter.

Figure 4B shows two dimer gene constructs, pX1D21c and pX1D22c. These constructs combine the ΔES1 and ΔNS1 mutated rDNA units, (fig. 1), into simple tandem arrays. The ΔNS1 mutation removes the spacer termination site (T), by fusing the first spacer promoter (SpPr I) with the pre-rRNA promoter (40S Pr). The ΔES1 unit is the "wild type" control gene used in the above experiments, see again fig. 1. On pX1D21c and -22c, any transcripts reading-through the ΔNS1 chimaeric promoter would be detected by more extensive protection of the pre-rRNA probe, than is afforded by just the pre-rRNA transcript, see fig. 4B and fig. 1. As expected, when these plasmids were microinjected, both showed readthrough of the ΔNS1 pre-rRNA promoter, (Readthr' in fig. 4A, tracks 1 and 3).

Two further dimer constructs, pX1D23c and -24c, were produced as exact parallels of pX1D21 and -22c, but in these the ΔES1 mutation was replaced by the slightly more extensive ΔESX1 mutation, fig. 4B. Since the ΔESX1 mutation inactivates the pre-rRNA promoter, if readthrough of the ΔNS1 chimaeric promoter on pX1D21c and -22c was solely due to promotion from the ΔES1 promoter, no such readthrough should be detected on pX1D23c and -24c. This is infact just what was observed, see tracks 2 and 4 in fig. 4A. (It should be noted that in this experiment the ΔES1 gene was coinjected on a separate monomer plasmid (pX1ΔES1c) as

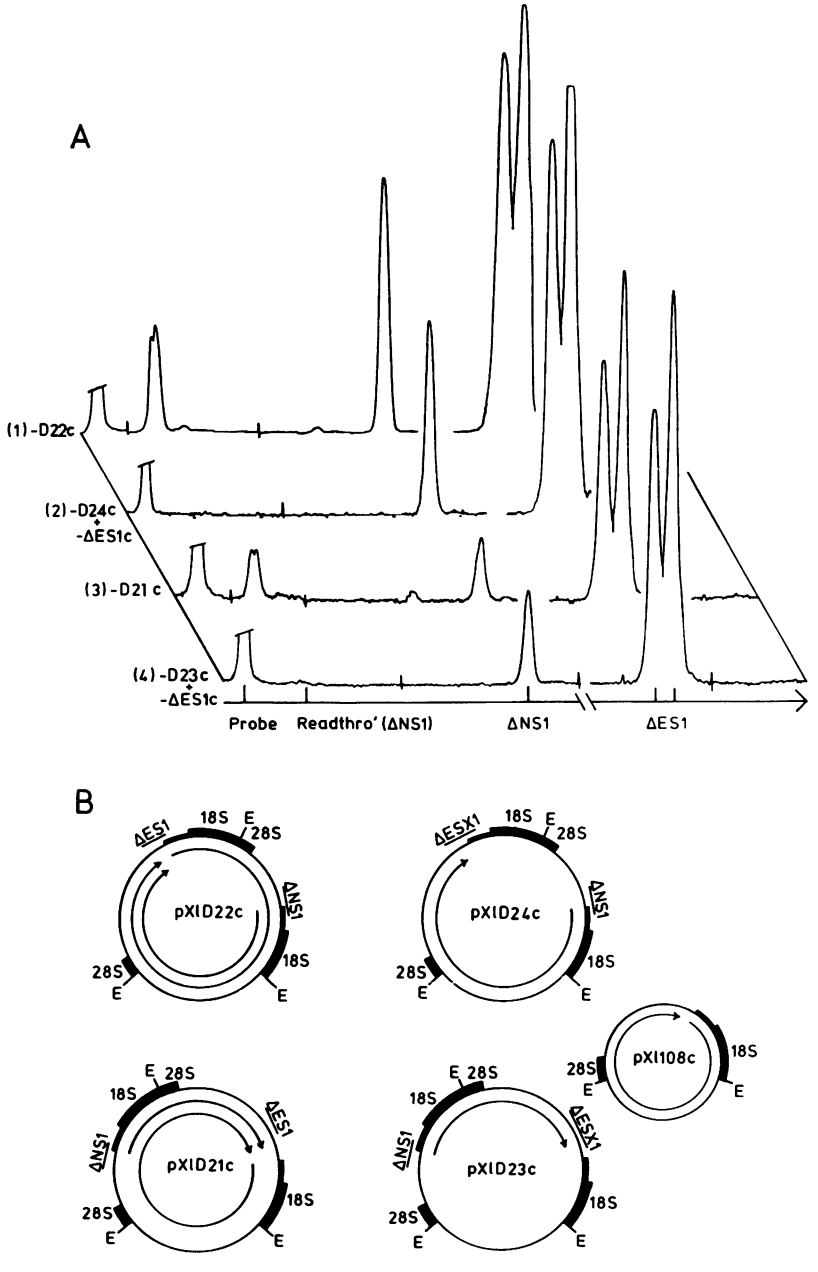


**Figure 4.** Transcripts produced on the microinjected rDNA plasmids. (A) S1-mapping of the transcripts on the dimer gene constructs pXlD21-24c. The results are shown as densitometer analyses of autoradiographs from a single

gel. Tracks 1 to 4 are respectively pX1D22c, pX1D24c coinjected with pX1ΔES1c, pX1D21c and pX1D23c coinjected with pX1ΔES1c. Each injection was analysed with the pre-rRNA probe, (~1x10$^6$cpm/pmole), (fig. 1). "Probe" refers to the position of the intact probe, "108", "ΔNS1" and "ΔES1" refer to the respective pre-rRNA signals, "Readthro'" refers to protection by transcripts reading through the ΔNS1 promoter. The ΔES1 signals are shown on a 2x greater density scale than the rest of the figure. (B) The dimer gene constructions. RDNA (EcoRI) inserts are identified as mutant or wild type as in fig. 1 and the 5'-ETS, 18S and 28S gene segments are indicated by thickened lines. Arrows inside the plasmid representations show the expected pre-rRNA transcripts. "E" shows the restriction sites for the enzyme EcoRI.

an internal control. Hence the ΔES1 signal is still seen in fig. 4A, tracks 2 and 4.).

Clearly, regardless of gene order, initiation at the ΔES1 pre-rRNA promoters of pX1D21c and D22c is the major, if not the sole source of the transcripts which read-through the ΔNS1 promoter, (compare in fig. 4A tracks 1 & 3 with 2 & 4). Hence no detectable level of transcription of the rDNA coding strand could have resulted from initiation events occuring within the region between the ΔES1 and ΔNS1 promoters on either pX1D21c or pX1D22c. Inspection of fig. 4B shows that for the former construct, this region is exactly equivalent to the transcribed region between the pre-rRNA promoter and the first spacer promoter of pX1108c. It can thus also be concluded that no significant level of transcription is initiated between these promoters on pX1108c. The only transcript reading into the 3'-ETS of the microinjected wild type rDNA construct is then that initiated at the pre-rRNA promoter.

**Most pre-rRNA transcription complexes make a complete transcript of the microinjected template.** Various experiments (1,8) have shown that the 3'-ETS does not significantly attenuate RNA polymerase I transcription of the microinjected rDNA. However the efficiency with which pre-rRNA transcription complexes traverse the vector region of rDNA constructs such as pX1108c has not been determined. If most pre-rRNA transcription complexes were to terminate or be attenuated within the vector, the results obtained when the pre-rRNA was prematurely terminated, could not be due to the inhibition of Readthrough Enhancement. The numbers of pre-rRNA transcripts running into and out of the vector on the rRNA coding strand were therefore compared.

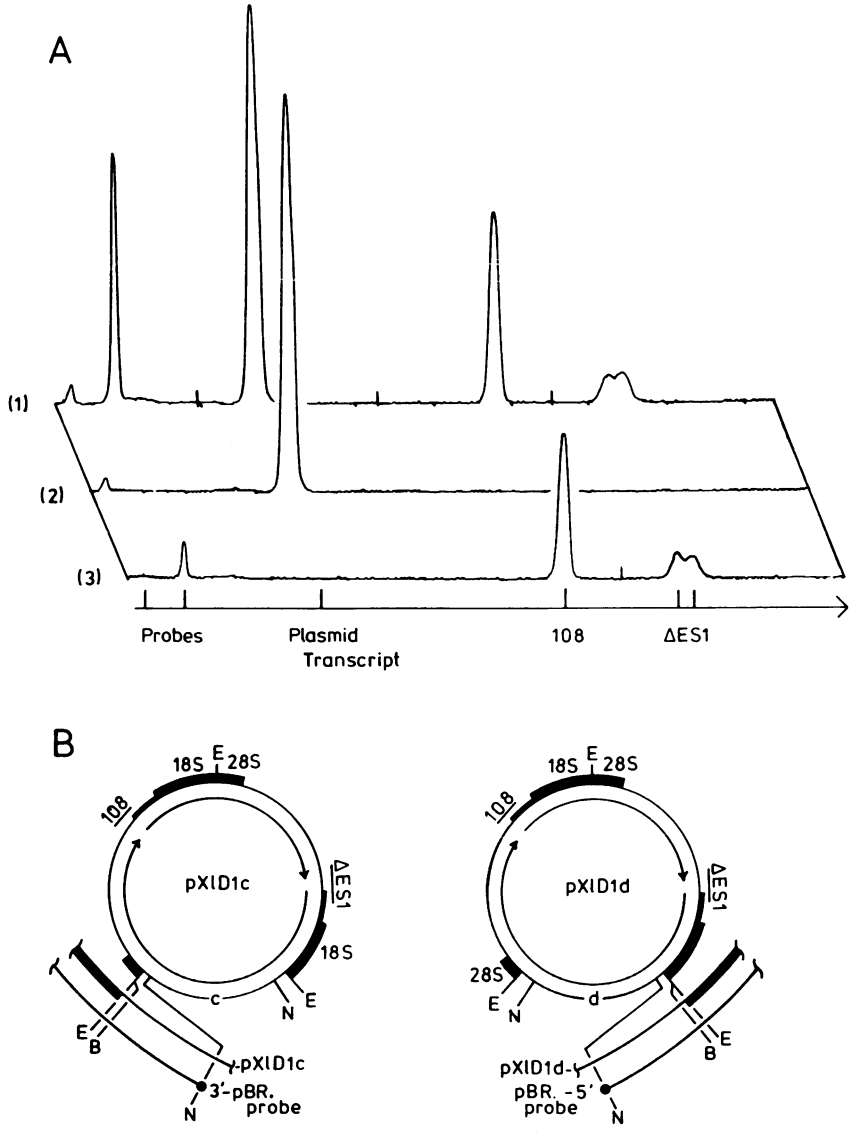


**Figure 5.** The pre-rRNA transcript is not attenuated within the vector. (A) S1-mapping of the transcripts on the dimer gene constructs pXlD21-24c. The results are shown as densitometer analyses of autoradiographs from a single gel. Track 1, pXlD1d analysed with both the 5' labelled pBR., ($1.5 \times 10^6$cpm/pmole), and pre-rRNA, ($0.3 \times 10^6$cpm/pmole), probes, and tracks 2 and 3 pXlD1c analysed respectively with the 3' labelled pBR. probe, ($1.3 \times 10^6$cpm/pmole), or with the pre-rRNA probe, see (B). "Probes" refers to the positions of the intact probes, "108" and "ΔES1" refer respectively to the pre-rRNA signals and "Plasmid Transcript" refers to protection of the

NciI-(BamHI)-EcoRI region of the pBR. probe, see (B). The ΔES1 signal is reduced in intensity since the hybridisation conditions chosen were optimum for the plasmid transcript. (B) The gene constructions used in (A) are shown along with an expansion of the regions probed for pre-rRNA transcription of the vector. RDNA (EcoRI) inserts are identified as control (ΔES1) or wild type (108) and the 5'-ETS, 18S and 28S gene segments are indicated by thickened lines. Arrows inside the plasmid representations show the expected pre-rRNA transcripts. The standard vector, (pBR-RIII), an EcoRI to BamHI deletion mutant of pBR322 is shown either in the "c" or "d" orientation. The S1-mapping probe derived from this vector is shown expanded, ("pBR. probe") and its labelling indicated (o-5' or o-3'). "B", "E" and "N" are restriction sites for the enzymes BamHI, EcoRI and NciI respectively.

The quantitative comparison of RNAs of different sequence by the S1-mapping technique, can in our hands lead to large errors, probably caused by the different stabilities of the hybrids formed. To avoid such uncertainties, two exactly analogous dimer gene constructs, each containing the wild type gene unit (108) and the essentially wild type ΔES1 control gene, were used. One construct in which the standard vector orientation ("c") was inverted, (pXlD1d in fig. 5B), was used in estimating the number of transcripts entering the vector. This number is independent of the vector orientation. The construct pXlD1c (standard vector orientation) was then used in estimating the numbers of transcripts exiting the vector. A single, vector derived, NciI - NciI probe, ("pBR. probes" in fig. 5B), either 3'-or 5'-end-labelled, was then used to detect and quantitate transcripts reading into and out of the vector, respectively on the "d" and "c" constructs. Since in both cases the RNA-DNA hybrids were of identical sequence, differing only in which strand was DNA and which RNA, the stability of each hybrid was essentially identical.

Transcripts reading out of the vector on the pre-rRNA coding strand of the dimer gene construct pXlD1c, (equivalent to pXl108c or pXlΔES1c for the purposes of this experiment, see fig. 5B) were detected using the 3'-end-labelled vector probe, fig. 5A track 2, ("plasmid transcript"). This signal was compared with the signal from the transcripts reading into the vector on pXlD1d, which were detected using the same probe, but this time 5'-end-labelled, fig. 5A track 1. After correcting for a 15% difference in specific activity between the 5' and 3' end labelling and normalising to the ΔES1 or 108 pre-rRNA signals for

both plasmids, fig. 5A tracks 1 and 3, the densitometric analysis revealed that the numbers of transcripts reading into and out-of the vector were the same +/-30%. Thus essentially all the pre-rRNA transcripts traversed the vector when it was in the standard "c" orientation. It is interesting also to point out that for the regions analysed in fig. 5A, no transcripts of the vector strand contiguous with the non-coding rDNA strand could be detected when the "d" construct was probed with the 3'-labelled pBR. probe or the "c" construct was probed with the 5'-labelled pBR. probe, (data not shown). Thus it is likely that little or no transcription of the non-coding strand occured in these experiments.

It can be concluded from the above experiments that on the microinjected rDNA plasmids pX1108c and pX1ΔES1c;

a) only one transcript, initiated at the pre-rRNA promoter, reads through the vector and into the 3'-ETS, i.e. exactly as expected for the tandem gene array found *in vivo*.

b) this transcript is only minimally, if at all, attenuated by the plasmid vector, 100 +/-30 % of transcripts reading through the vector.

Thus the inhibition of pre-rRNA initiation noted when the RNA polymerase I terminator was inserted into the 18S gene, fig. 3, must have been a direct result of prematurely terminating the pre-rRNA transcript.

## DISCUSSION

This study has shown that premature termination of the *X. laevis* pre-rRNA transcript causes an 80 to 90% inhibition in the promotion of this same transcript on circular microinjected templates. Extensive control experiments argue that this inhibition must be a direct result of the premature termination. Firstly, only one transcript, the pre-rRNA, is made on the coding strand of the microinjected template. Secondly, in the absence of premature termination, this transcript is complete, being terminated at the *in vivo*, pre-rRNA promoter proximal site, "T" in fig. 1. The site chosen for premature termination was distant from both the 3'-ETS and the pre-rRNA promoter, being 1720 b.p. downstream or ~9040 b.p. upstream of the pre-rRNA initiation site

on the circular template. It is therefore very unlikely that the insertion of an extra terminator at this site significantly affected the intrinsic activity of the pre-rRNA promoter or the 3'-ETS enhancer elements. The fact that the insertion of an inverted terminator sequence at the same site had no affect on pre-rRNA initiation, further supports this conclusion. The stability of the pre-rRNA transcript was also not significantly affected by premature termination. The observed inhibition of pre-rRNA initiation must therefore be interpreted as being a direct result of prematurely terminating the pre-rRNA transcript.

RNA polymerase I normally transcribes the 3'-ETS to within 213 b.p. of the pre-rRNA initiation site, i.e. within ~4 polymerase diameters of the upstream boundary of the pre-rRNA promoter, see fig. 1. It is therefore likely that by prematurely terminating its transcription, recapture and hence recycling of the polymerase or other transcription factors, by the pre-rRNA promoter, was inhibited. Thus on the wild type microinjected template, pre-rRNA transcription positively feeds-back to enhance its own promotion. This mechanism has been called "Readthrough Enhancement" since, to occur *in vivo*, it would require transcriptional readthrough from one cistron to the next, (6). The present study provides the first evidence for "Readthrough Enhancement" in *X. laevis* and provides a more direct test of this phenomenum than has as yet been possible in other systems. On mouse rDNA, mutation of the pre-rRNA promoter proximal termination site, caused an inhibition of pre-rRNA promotion in cell transfection experiments, (10,11). However this also lead to transcripts reading through the pre-rRNA promoter. Thus promoter occlusion may have been the cause of the observed effect. Similar experiments on the *X. laevis* rDNA, but performed in cell extracts, were unable to detected an effect attributable to Readthrough Enhancement, (9). The fact that we do see such an effect on the same genes, may simply reflect the difference between the *in vivo* and *in vitro* assay.

In order to test for Readthrough Enhancement in a near *in vivo* situation, the present experiments were performed with a complete, wild type rDNA spacer. The presence of the spacer promoters (SpPr in fig. 1) may at first sight appear to

compromise our conclusions, since their transcripts were not prematurely terminated along with the pre-rRNA transcript. As discussed in the introduction, recent experiments (5) have shown that spacer promoter activity, while being essential for efficient gene activity, does not have any effect when the 60/81 b.p. enhancer elements have been deleted. Thus spacer promoter transcription has no significant direct effect on pre-rRNA transcription. We have postulated that the spacer promoters may be involved in some step of enhancer activation (5). Experiments in progress further show that inactivation of the spacer promoters has no effect on Readthrough Enhancement and suggest that the level of spacer promoter transcription is normally significantly less than that of the pre-rRNA promoter, (Moss and Larose, unpublished results).

The data presented here cannot provide an absolute demonstration of Readthrough Enhancement. However few other explanations are consistent with the observed effect being a direct result of premature pre-rRNA termination. One valid alternative is that transcription of the 3'-ETS could be necessary to maintain the active state of the enhancer elements and the promoter itself, e.g. by maintaining an "open" chromatin configuration in this region. A further alternative, is that the site at -213 b.p. does not terminate the pre-rRNA transcript, but processes it by a combination of endo-and exonucleolytic activities, to produce a 5' terminus indistinguishable from that of the newly initiated pre-rRNA. This is not very likely in view of the various reports on pre-rRNA termination, (1,7,8). However the apparent homology between one consensus block of the terminator and a known pre-rRNA processing site (7), means that such a possibility, perhaps in combination with true termination, must at least be kept in mind.

Since transcription of the *X. laevis* ribosomal genes has been shown to be enhanced by sequences in the 3'-ETS, how might Readthrough Enhancement fit into the overall picture of the expression of these genes? The observations presented here, (i.e. 80 to 90% inhibition of promotion when the pre-rRNA is prematurely terminated), if interpreted in terms of this mechanism, would suggest that 80 to 90% of the polymerase molecules are

recycled. We perceive the 3'-ETS enhancer sequences then to correct this deficit, by attracting polymerase and/or other factors from the free nucleolar pool. Thus a consistently high level of transcription is maintained by the combination of the two effects.

Readthrough Enhancement would be most obviously useful on tandemly repeated genes, such as the ribosomal genes, where high levels of transcription must be maintained. However it is quite feasible that tandemly arranged single copy genes might also use this mechanism to co-ordinately regulate their expression. Studies with tandemly arranged RNA polymerase II genes have suggested that readthrough from an upstream gene, if not terminated, can repress transcription from the downstream gene, (15). We suggest the converse could also occur, that is, the expression of a downstream gene could be enhanced as a direct result of the activity of one immediately upstream. This linkage of expression could be regulated by switching the site for termination of the upstream gene. Termination at a site distal from the downstream gene promoter would not enhance expression of this gene, whereas termination at a proximal site would.

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