Promoter efficiency depends upon intragenic sequences

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ABSTRACT
Experiments concerning gene transcription in Xenopus oocytes have revealed that the efficiency of the HSV-TK promoter is dependent upon the nature of the attached gene sequences. Transcriptional efficiency of the TK promoter when attached to its own gene was 30-fold higher than that observed when the promoter was attached either to an avian keratin gene or to the chicken histone H2B gene. Furthermore, attachment of the keratin gene promoter to the TK gene resulted in a 20-fold increase in keratin promoter efficiency. It was found by subsequent experiments that the TK gene is able to exert a stimulatory influence upon attached promoter sequences. This cis effect was shown to be independent of orientation but dependent upon the distance between the DNA sequences involved.

INTRODUCTION
Studies of gene expression frequently involve the attachment of a readily detectable marker gene to a gene promoter under study (1-10). Such chimaeric constructs provide a convenient way to detect promoter activity in a quantitative manner. Similarly, structural gene sequences under study have been linked to promoters of well defined characteristics (11-13). In order to study aspects of feather keratin gene transcription, we attached the keratin coding sequences (14) to a promoter of known efficiency, the Herpes simplex virus thymidine kinase (HSV-TK) gene promoter. The essential elements of the HSV-TK promoter have been examined in detail and its efficiency in directing transcription within the Xenopus oocyte is well established (15,16). In the course of these experiments, we have obtained results that add to the growing body of evidence that promoter function may be dependent upon DNA sequences in diverse locations (17-22). We also demonstrate here that the efficiency of a gene promoter in the Xenopus oocyte can depend heavily upon the nature of the gene sequences to which it is attached.

METHODS
Oocyte injection
The micro-injection technique and the conditions used for oocyte culture have been described (23). In each experiment, batches of 30-50 oocytes were injected. Unless
otherwise stated, each oocyte nucleus received 5 ng of test DNA and also (when necessary) 5 ng of p7AT histone DNA (see below) in a total injection volume of 50 nl. After incubation at 18°C for 24 hours in Barths’ saline, total nucleic acid was extracted (24).

Histone gene clone p7AT

The clone p7AT contains a cluster of three chicken histone genes (H1, H2A and H2B; ref. 25) This clone was often injected with the test DNA templates and primer extension assays of histone gene transcripts served as an internal control.

Gene Constructs

The techniques used in the manufacture of the recombinant plasmids below were as described in Maniatis et al. (26). The manufactured constructs were checked by restriction endonuclease mapping and dideoxy sequencing (27). For the sake of brevity, all of the specific details relating to the manufacture of each construct have not been given. Diagramatic representations of each construct appear in figures showing the results of injecting that construct into oocytes.

TK clone

The manufacture of this clone has been described previously (14) and it contains the entire HSV-TK gene in a 3.4 kb Bam HI fragment which has been inserted into the Bam HI site of pBR322.

KER

This clone is a modified avian keratin gene (B) from which the single intron, located in the 5’ untranslated region, has been removed. The construction of this clone has been described previously (14).

KER/TK

In KER/TK the keratin promoter (a 399 bp Pst I fragment spanning positions -354 to +45, ref. 28) is fused to the 2.7 kb Bgl II/Bam HI fragment of the HSV-TK gene (15). The size of the clone is 7.12 kb.

TK/KER

This construct contains the TK promoter (a 680 bp Bam HI/Bgl II fragment, ref. 15) fused to keratin gene B coding and 3’ untranslated sequences (14). The construct contains 31 bp of pBR322 DNA between the junction of the TK promoter fragment and the keratin gene sequences. The size of the clone is 5.78 kb.

MTK/KER

In this construct the TK promoter was fused to keratin gene sequences, however, MTK/KER lacks the 31 bp pBR322 DNA referred to above. MTK/KER was used as the basis for a number of deletion constructs.

MTK/KER-BB

MTK/KER-BB is a derivative of MTK/KER and lacks a 316 bp Bst EII fragment from the keratin protein coding region.
MTK/KER-SmB

This clone is also derived from MTK/KER and has a 346 bp Bst Ell/Sma I fragment deleted from the keratin coding region.

MTK/KER-SSm

All of the keratin sequences 5' to the Sma I site in the coding region of gene B (870 bp) were removed by digestion with Sma I and Hind III. A 250 bp Pvu II/Bgl II fragment, containing the TK promoter (15) was inserted, generating MTK/KER-SSm with a size of 5.25 kb.

BKTK5 and BKTK2

These constructs contain a 1.7 kb Bgl II/Pvu II fragment from the TK gene (15) inserted into the Sma I site of KER. In BKTK5, the TK fragment is in the functional coding sense orientation relative to the keratin promoter. The TK fragment in BKTK2 is inserted in the opposite direction to that in BKTK5. The size of both constructs is 7.2 kb.

TK/H2B and KER/H2B

An M13 clone, M13H2BX-X, containing the 1.85 kb coding region of the chicken histone H2B gene (29) inserted in the Hinc II site of M13mp19 was used as a vector to insert the TK and keratin promoter sequences. M13H2BX-X was digested with Sma I. To construct TK/H2B, the 250 bp Pvu II/Bgl II fragment containing the TK promoter (15) was inserted into Sma I site of M13H2BX-X. To construct KER/H2B the 399 bp Pst I fragment from feather keratin gene B containing the keratin promoter was inserted into the Sma I site. The size of TK/H2B is 9.35 kb and the size of KER/H2B is 9.5 kb.

Spacer constructs

In these constructs, the keratin promoter was separated from the TK gene by varying lengths of keratin DNA. In the case of KER436TK, the spacer DNA was a pBR322 fragment. The Pst I fragment containing the keratin promoter (see KER/TK) was inserted into the Pst I site of M13mp18. Various fragments of keratin DNA were inserted into restriction enzyme sites downstream of the Pst I site in the polylinker region. These extended keratin promoters were resected from M13mp18 and fused to the coding region of the TK gene to generate the following constructs, KER127TK, KER187TK, KER328TK, KER430TK, and KER436TK. The central number of each construct name represents the distance in base pairs from the cap site in the keratin promoter to the ATG in the coding region of the TK gene.

Primer extension and measurement of transcriptional efficiency

Primer extension analysis (30) was used to map the 5' ends of the various gene transcripts. Typically, 1.5 ng of kinased synthetic primer was precipitated with the RNA equivalent of two oocytes. Primers were annealed to the RNA at 42°C for 1 hour. The extension reaction used AMV reverse transcriptase in the presence of all four deoxynucleotide triphosphates (0.6 mM). Reaction products were electrophoresed on
Linkage of the keratin promoter to the TK structural gene results in a high level of transcription. Oocytes were injected with either TK (intact TK gene, track 3), KER/TK (keratin promoter fused to the TK gene, track 4), TK/KER (TK promoter fused to the keratin gene, track 5), or KER (keratin gene B with its intron removed, track 6). An equal amount of p7AT DNA containing the chicken histone H1 gene was included as an internal control. In tracks 3a to 6a, primer extensions were performed on the same RNA as that in tracks 3 to 6 except an H1 histone specific primer was used. A diagrammatic representation of the constructs is shown under the autoradiograph, the small bars depict primer location.
denaturing polyacrylamide gels (27). For the purposes of densitometric scanning, X-ray films were flashed before exposure (31). Densitometry traces were performed using an LKB 220Z Ultrascan Laser densitometer with integrator. The efficiency of transcription was determined by densitometric scanning of a primer extension product band where the RNA from two injected oocytes was used in the extension reaction.

**Synthetic primers**

Synthetic primers (Biotechnology Research Enterprises S.A. Pty. Ltd.) were used to detect gene transcripts. The approximate location of the primers has been indicated in diagrams representing each injected construct.

**Measurement of RNA accumulation in oocytes and determination of the rate of RNA degradation**

The procedures used were essentially those described by Miller *et al.* (32) except that gridded nitrocellulose filters were used and the hybridizations were performed in plastic bags at 42°C.

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![Graph](image-url)

**Fig. 2.**
Disappearance of labelled RNA under α-amanitin chase conditions. Oocytes were injected with 10 ng of one of four constructs: KER (○), TK/KER (△), KER/TK (●) or TK (■). One µCi of α-32P-rGTP was co-injected with each DNA template. The oocytes were then incubated for 24 hours. Each oocyte was reinjected with 10 nl of α-amanitin (100 µg/ml). Batches of 10 oocytes were removed at varying times after the α-amanitin injection and the amount of labelled transcript remaining was determined by hybridization to nitrocellulose bound DNA. The points represent an average of two experiments and the data was normalized to the amount of radioactive transcript present at the time of addition of α-amanitin.
RESULTS

The HSV-TK gene was transcribed efficiently in Xenopus oocytes with a yield of mRNA approximately 30-fold greater than that observed for feather keratin gene B (fig. 1). In these experiments the feather keratin gene intron was not present and its known, inhibitory effect could therefore be disregarded (14).

When the HSV-TK gene promoter was attached to the keratin gene (construct TK/KER) there was no detectable increase in the production of keratin mRNA (fig. 1). However, attachment of the keratin gene promoter to the coding region of the TK gene (construct KER/TK) resulted in transcription at an efficiency close to that of the unmodified TK gene (fig. 1).

The possibility that the mRNA products may have been detected at different levels because of differing mRNA stabilities was tested by measuring the half life of each transcript, following α-amanitin treatment of the injected oocytes (fig. 2). No significant difference was detected in the rates of mRNA degradation. It was concluded that the differences observed in the levels of mRNA were the result of different transcriptional efficiencies.

Two major possibilities were considered, as explanations for these observations.

1. Keratin gene sequences may exert an inhibitory influence upon the associated promoter, as the intron sequences had previously been shown to do (14).

2. TK gene sequences may exert a stimulatory influence upon attached promoters.

Deletion of keratin gene sequences to test for inhibitory effects

A series of gross deletions, within the protein coding and 3' untranslated region of the keratin gene produced no significant change in transcriptional efficiency under control of the TK gene promoter (fig. 3). It was concluded that no specific part of the keratin DNA sequence could be responsible for the transcriptional inhibition.

The possibility that any of the attached keratin gene sequences, rather than a particular keratin sequence may have had an inhibitory effect on TK promoter efficiency was eliminated in the following experiment. The TK promoter was fused to the coding region of the H2B histone gene, generating TK/H2B and in the process effectively eliminating all keratin gene sequences. The H2B histone gene is transcribed efficiently in oocytes under the control of its own promoter (fig. 6 and ref. 14) and also other gene promoters (33). However, the efficiency of transcription from TK/H2B was low and comparable to the level of transcription from KER/H2B in which the keratin promoter was fused to the H2B gene (fig. 4). Transcription from the intact TK gene was again 30-fold greater than that observed from either TK/H2B or KER/H2B. It was concluded that keratin gene sequences were not solely responsible for the low levels of transcription from the TK promoter.

Stimulation of transcription by TK gene sequences

The remaining possibility that TK gene sequences might provide a stimulatory
Fig. 3.
Deletions in the keratin sequences of TK/KER. Oocytes were injected with constructs derived from TK/KER. Each construct except MTK/KER had a deletion in the keratin sequences. An equal amount of p7AT DNA was co-injected with each construct except TK and primer extensions using the H1 primer acted as an internal control (indicated). A representation of each construct is given below the autoradiograph. The bars show the location of the primers used in the extension reactions. The symbol (•) indicates extension products derived from cap site initiation and (t) indicates transcripts initiating from the TATA box of the injected construct (see ref. 14). S=Sal I, Sm=Sma I, B=Bst EII and C=cap site location.
influence upon transcriptional promoters was directly tested by inserting the entire protein
coding and 3' untranslated region of the TK gene into a unique restriction site in the keratin
coding region, generating construct BKTK5. This modification allowed the keratin
promoter, attached to the chimaeric gene, to function with an efficiency identical to that
observed with KER/TK, thus approaching the efficiency of the intact TK gene. Furthermore,
a second construct (BKTK2) with the TK gene inserted in the opposite orientation, was also
transcribed with increased efficiency (fig. 5).
Effect of inserting the TK gene (minus promoter) into KER. BKTK5 and BKTK2, containing the 1.7 kb Bgl II/Pvu II TK gene fragment inserted into the Sma I site of KER were injected into oocytes. KER/TK was also injected for comparison. Diagrams below the autoradiograph show each construct with the arrows indicating the orientation of the TK gene. The bars show the location of the primer used.

To test whether the TK stimulatory effect was distance dependent, constructs were made containing extra fragments of keratin coding sequence between the keratin promoter and the TK gene (fig. 6). It was shown that an increase in the distance between the keratin cap site and the ATG of the TK gene from an original 101 bp to 430 bp resulted in a reduction in transcript levels. High levels of transcript were still observed when 328 bp of keratin spacer DNA was present between the keratin cap site and the protein initiation codon of the TK gene (fig. 6).

We concluded that the thymidine kinase gene contains DNA sequences that are
Fig. 6.
The effect of separating the keratin promoter from TK gene sequences. Oocytes were injected with a number of constructs in which the spacing between the keratin promoter and the TK coding region was progressively increased. These constructs are depicted underneath each autoradiograph in panels A and B. Each construct is numbered and the number corresponds to the appropriate track in the autoradiograph. The central number of the construct name denotes the distance from the cap site of the keratin promoter to the ATG of the TK gene. The bar shows the location of the primer. Oocytes used in panel A were obtained from a different frog to those used in B. All constructs in panel A were co-injected with p7AT. In tracks 2a to 6a of panel A, primer extensions were performed on the same RNA as that used in tracks 2 to 6 except that an H2B specific primer was used. M = Hpa II cut pBR322 markers, (*) represents transcript initiation from the cap site of each construct and (>) represents transcripts initiating from the TATA box (see ref. 14).
capable of exerting a stimulatory effect upon transcription from attached promoters in the Xenopus oocyte. This cis-effect was shown to be independent of orientation but dependent upon the distance between the DNA sequences concerned.

Preliminary attempts to locate the stimulatory region within the TK gene, involved inserting large fragments of the TK gene into the same location within the keratin gene. So far, none of the fragments tested have caused transcriptional stimulation (data not shown).

DISCUSSION

The results obtained in this study raise two interesting and important points. First, the construction of hybrid genes to obtain information must be used very cautiously. It has been common practice to attach indicator genes such as the CAT gene (1-5) and the TK gene (6-8) to foreign gene promoters in order to study the functioning of those promoters. It is now clear from the data obtained here and elsewhere (17-22) that the behaviour of a gene promoter can be heavily dependent upon the gene to which it is linked.

Secondly, the primary DNA sequence within a gene promoter may be replacable by apparently unrelated sequences, with little loss of functional efficiency. Experiments by McKnight et al. (16) using deletions to examine which parts of the TK gene promoter are essential for normal function showed clearly that specific parts of the promoter sequence must be present for efficient transcription to occur. In our own laboratory, removal of the second distal element of the TK promoter resulted in a 90% reduction in promoter efficiency (14) as was also described by McKnight et al. (16). However, when the TK promoter was replaced by the feather keratin gene promoter, transcription occurred at approximately 70% of normal efficiency. Examination of the DNA sequence of the two promoters reveals almost no similarity between them apart from the canonical TATA box. Thus it appears that the essential elements of the TK gene promoter can be substituted by DNA sequences that bear no resemblance, in terms of primary structure. It must be assumed that different factors are responsible for the activity of the TK and keratin promoters in Xenopus oocytes.

The nature of the interaction between the TK gene sequences and the keratin promoter attached to it remains unknown. The characteristics of distance dependence and orientation independence suggested to us that the activatory sequences involved could be almost centrally located within the TK gene. The absence of any major palindromes within the TK gene rules out the possibility of symmetry in primary sequence as a reason for orientation independence. Unfortunately, attempts to evoke the transcriptional stimulation, using large sub-fragments of the TK gene, inserted into the keratin coding region, have been unsuccessful. It is possible that the stimulatory sequences involved could have been disrupted by the fragmentation of the TK gene in these experiments. Finer deletion mutants need to be constructed in order to define the activatory sequences within the TK gene.
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