KTF-1, a transcriptional activator of Xenopus embryonic keratin expression

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Summary

Nuclear extracts from embryos of Xenopus laevis were shown to contain a protein activity, KTF-1, which binds in vitro to the promoter of the embryonic, epidermis-specific keratin gene, XK81A1. Mobility shift assays, methylation interference and footprinting analysis were used to show that the KTF-1 binding site contains an imperfect, palindromic sequence, ACCCTGAGGCT. This sequence occurs once in the XK81A1 promoter, 152-162 base pairs upstream of the transcription start site. A construct of the keratin gene in which this sequence was altered so that it no longer binds KTF-1 in vitro showed severely reduced transcription levels upon injection into Xenopus embryos, but retained epidermal specificity. Addition of KTF-1 binding sites also enhanced epidermal and non-epidermal activity of a heterologous promoter, Xenopus β-globin, in embryos. These results suggest that KTF-1 is a general activator of embryonic keratin transcription, which acts in concert with other factors to produce high levels of epidermis-specific expression.

Key words: DNA binding protein, embryonic keratin, transcriptional activator, Xenopus.

Introduction

The XK81A1 embryonic epidermal keratin gene of Xenopus laevis is an example of a tissue-specific gene that is regulated in response to early developmental events. At mid-blastula transition it undergoes rapid, cell autonomous activation in the outer ectoderm (Sargent et al. 1986; Jamrich et al. 1987), but it also shows response to induction; in vivo it is turned off by neural induction (Jamrich et al. 1987), and in vitro can be deactivated by growth factors, such as XTC-MIF, which convert ectoderm to mesoderm (Symes et al. 1988). Thus, a knowledge of the transcription factors that control XK81A1 expression should lead to greater understanding of both mosaic (cell autonomous) and regulative (cell interactive) developmental decisions.

Previously, we used injection of the cloned keratin gene into fertilised Xenopus eggs to identify regions of the gene that are necessary for tissue-specific expression (Jonas et al. 1989). We showed that the coding and 3' sequences of the gene, downstream of +26, could be substituted by a reporter gene (human β-globin), and that 5' flanking sequence could be deleted to −487, without loss of epidermis-specific expression. Further 5' deletions resulted in progressive loss of regulation, so that expression was both reduced in epidermis and increased in non-epidermal tissues. This suggests that both positive and negative transcription factors sufficient for correct regulation of this gene bind sequence elements between −487 and +26. Our next aim is to identify these elements and the embryonic proteins that recognise and bind them.

In this paper, we report on the identification of a DNA-binding activity, KTF-1, from tailbud stage Xenopus embryos, which recognises a specific sequence centred around position −157 in the XK81A1 promoter. In vitro mutagenesis was used to create a gene in which the KTF-1 binding site is non-functional, and this was injected into embryos. The results of such experiments support a role for KTF-1 as a transcriptional activator which increases XK81A1 expression in epidermis. However, the mutated gene, which lacks the KTF-1 binding site, is still expressed, to a lower level, specifically in epidermis. This suggests that, like other regulated genes, XK81A1 is controlled by a number of transcription factors, including KTF-1.

Materials and methods

Nuclear extracts

Nuclear extracts from 300 to 3000 tailbud Xenopus embryos, stages 17–22 (Nieuwkoop and Faber, 1967), were prepared according to the method of Mohun et al. (1989), with modifications: the cell homogenisation buffer contained 2.2 M sucrose, and was supplemented with 1% no-fat, dry milk; only a single centrifugation was used to purify nuclei. After lysis of the nuclei in 0.55 M KCl, the chromatin was pelleted as described (Mohun et al. 1989), and the supernatant concen-
trated in Centricon 10 microconcentrators (Amicon, W. R. Grace and Co., Danvers, MA). It was then diluted with potassium-free buffer (26 mM Hepes pH 7.6, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM PMSF), to a final KCl concentration of 40 mM. The diluted supernatant was then reconstituted to give approximately 4 embryo equivalents per μl of the final extract.

DNA mobility shift assays
Each 15 μl binding reaction contained 50 mM NaCl, 0.1 mM EDTA, 20 mM Hepes pH 8, 5% glycerol, 10 μg ml⁻¹ BSA, 4% Ficoll-400, 0.004% bromophenol blue, 0.004% xylene cyanol, with DTT added to 1 mM just before use, 5 μg double-stranded poly(dI.dC).poly(dI.dC) (Pharmacia) as non-specific competitor, 0.1 ng end-labelled, double-stranded DNA probe (approximately 10⁸ cts min⁻¹, see below), specific competitor DNA, as appropriate, and 0.5–5 μl nuclear extract, added last. Specific competitors were isolated restriction fragments of plasmid DNA, their concentration estimated by ethidium bromide staining in agarose gels, or double-stranded synthetic oligo-deoxyribonucleotides. Double-stranded oligo competitors were prepared by annealing complementary single-stranded oligos at 0.1 mg ml⁻¹, in 200 mM NaCl, for 30 min, at 37°C. After precipitation and resuspension, the DNA concentration was checked by measuring the OD₂₆₀. The binding reactions were incubated at room temperature for 45 min, then loaded directly onto a 3.5% acrylamide gel (acrylamide: bis-acrylamide ratio=19:1) which was run in 50 mM Tris, 20 mM boric acid, 10 mM EDTA pH 8.8. Gels were dried and exposed to X-ray film.

Probes
To make probe 1, the −423 to −140 fragment of XK81A1 was subcloned into pBS+ (Stratagene). For labelling of the coding strand, the subclone was linearised using Bsll, cutting with BglII, at −274, and end-labelled using polynucleotide T4 kinase (Baker and Howley, 1987). The probe fragment was then released by digestion at —274, and —150, as shown in Fig. 5, using the Klenow fragment of DNA polymerase I and [32P]dGTP (Ausubel et al. 1987). End-labelled probe 1 was partially methylated by DMS (Ausubel et al. 1987), and used in scaled up binding reactions (1 ng probe, 30 μg poly(dI.dC), 6–10 μl nuclear extract). Free and bound probe were separated on an acrylamide gel, as for mobility shift assays, and then electroblotted onto NA45 DEAE-cellulose paper (Schleicher and Schuell). The bands were located by autoradiography, and the DNA eluted and piperidine cleaved as described (Ausubel et al. 1987) before analysing on 6% sequencing gels. A standard Maxam and Gilbert (1980) A/G sequencing analysis of probe 1 was run in parallel (Ausubel et al. 1987).

Results

DNA–protein binding studies
DNA mobility shift assays (Fried and Crothers, 1981) were used to look for evidence of proteins binding the XK81A1 promoter. Nuclear extracts prepared from early tailbud Xenopus embryos, which are actively expressing the gene, were found to contain an activity that specifically binds a BglII–HinFI fragment, from −274 through −140 (probe 1; Fig. 1). The binding is competed by 500X molar excess of unlabelled probe, but not by a non-specific competitor, the 676 base pair (bp) RsI fragment of pUC19. The shift was abolished by preincubation of the nuclear extract with proteinase K (not shown), indicating that the DNA-binding activity depends on a protein, or proteins. When the probe fragment was divided into two competitor fragments, of 59 and 80 bp, the upstream fragment (2) did not compete, while the downstream fragment (3) competed as efficiently as the whole probe sequence, indicating that the protein-binding site lies within fragment 3.

In order to identify the binding site more precisely, we made a series of overlapping, 30 bp oligodeoxyribono-
Fig. 1. Nuclear extract from Xenopus embryos contains an activity that binds the keratin promoter. Diagrams show the restriction fragment probe 1, and competitor sub-fragments 2 and 3 (see text). Solid lines represent keratin promoter sequence; the broken line represents 4 bp of vector sequence. Relevant restriction sites are shown, with their positions in the XK81A1 promoter: Bg, BglII; H, HindIII (not regenerated in subcloning); R, Rsal (in vector); X, XmnI. DNA mobility shift assays: each lane contains an equal amount of end-labelled probe 1, poly(dI.dC) competitor, and nuclear extract from tailbud embryos (see Materials and methods). In the first lane, no specific competitor was added; in subsequent lanes, unlabelled competitor, and nuclear extract from tailbud embryos (see Materials and methods). In the first lane, no specific competitor was added; in subsequent lanes, unlabelled competitor was used in 5, 50 and 500-fold molar excess, as shown. pUC(676R) is a 676 bp, Rsal fragment of pUC19 (unrelated competitor). In each lane, the upper band is protein bound, retarded probe and the lower band is unbound probe.

nucleotides covering the sequence of fragment 3, and used them as unlabelled competitors of probe 1 in the mobility shift assay. These data are summarised in Fig. 2A. Only oligo c (-165 to -136) gives competition, whereas the oligos which overlap c (f and g) do not, suggesting that a binding site lies within c, around the junction of f and g. This was confirmed by making a set of variants on oligo c, each one having a small number of altered base pairs (Fig. 2B). Only alterations in the first sixteen base pairs abolished the ability of the oligo to compete for binding (Fig. 2C). This region includes an imperfect palindromic sequence of eleven bases, ACCCTGAGGCT, centred around the G residue at position -157. Transcription factors often bind to imperfect palindromic sequences (Curran and Franza, 1988); however, this sequence does not appear to correspond to the binding site of any previously identified factor (see Discussion). We were able to show that this sequence can act as a positive regulatory element for keratin gene transcription (see below). We therefore named the embryonic DNA-binding activity KTF-1, for Keratin Transcription Factor-1.

DMS-methylation interference and footprinting analysis were used to obtain more information on the DNA-KTF-1 interaction. DMS-interference identifies G residues, methylation of which interferes with binding of a protein to DNA (Siebenlist and Gilbert, 1980). As shown in Fig. 3, KTF-1 binding to probe 1 is reduced by DMS methylation of either of the GG pair at -155 and -156 on the coding strand, or any of the three Gs at -159 through -161 on the non-coding strand, suggesting that all these residues are directly involved in protein binding. However, methylation of the central G at -157, or of the G at -153 (which constitutes an imperfection in the palindromic repeat), does not interfere with KTF-1 binding, implying that these residues are not directly associated with the protein.

In 1,10-phenanthroline–copper ion (OP-Cu) footprinting (Kuwabara and Sigman, 1987), protein binding protects the DNA from cleavage by this reagent. As shown in Fig. 4, KTF-1 binding to probe 1 protects residues -166 through -148 on the coding strand, and -166 through -148 on the non-coding strand. On the non-coding strand, residues -166 through -169 are also partially protected, while on the coding strand, KTF-1 binding appears to create a site, at residue -147, which is hypersensitive to OP-Cu digestion. The information from methylation interference and footprinting is summarised in Fig. 4B. These results are in good agreement with the data from mobility shifts.

Functional analysis of KTF-1 binding

The role of KTF-1 in the control of keratin transcription was investigated by combining in vitro mutagenesis with an embryo injection assay. Our previous analysis (Jonas et al. 1989) had shown that 487 bp of the upstream promoter is sufficient to confer epidermis-specific expression on an injected XK81A1 gene (K487, see Fig. 5). We therefore constructed a mutated version of K487, M157E (Fig. 5), replacing nucleotides -165 to -136 with oligo c5 (Fig. 2B), so that the KTF-1 binding site is altered, and no longer competes for binding in the mobility shift assay (Fig. 2C). We compared the promoter activity and tissue specificity of K487 and M157E, by injecting them into fertilised Xenopus embryos, allowing the embryos to reach tailbud stage, and then analysing expression from the injected genes by RNase protection assay, as previously described (Jonas et al. 1989).

For the analysis of promoter strength, the two constructs were injected separately into sibling embryos. A reference construct, KG487 (Fig. 5), which is a fusion of the unaltered keratin promoter (from -487 to +26) with the coding sequence of human β-globin, was mixed and coinjected with each of the test genes. In each experiment, filter hybridisation of DNA from the
Fig. 2. Mobility shift competition analysis allows location of the protein binding site within probe 1. (A) Overlapping 30mers a–g cover the sequence of competitor fragment 3 (see Fig. 1). Competitors a, b and c cover the XK81A1 promoter sequence from −225 through −136, while e, f and g are offset by 10bp, and cover −215 through −126. Competitor d is vector sequence, including the 4bp in probe 1 and fragment 3. In the mobility shift analysis (not shown) only c competes with probe 1 for binding at 50–500× molar excess. (B) Localisation of the protein binding site within c. Competitors c1–c7 contain altered base pairs (underlined). Competitors c6 and c7 have additional GATC linker sequences (bold type). Where the mutation alters the protein binding site (c1, c2, c5, c7), competition with probe 1 is abolished. (C) Results of mobility shift competition analysis. Mobility shift on probe 1 was carried out as in Fig. 1. Excess unlabelled oligonucleotides were used as competitors (comp.) at the molar ratios indicated.

injected embryos against probes specific for either globin or keratin showed that the control and test injected DNA survived and replicated to a similar extent. Thus it is valid to normalise expression of the test genes against KG487 expression, which was assayed by a globin-specific RNase protection probe (Karlsson et al. 1988). By this method, we found that mutation of the KTF-1 binding site significantly lowers the level of transcription from the injected keratin construct. A sample set of results is shown in Fig. 6A.

The experiment was repeated several times, using three separate preparations of M157E and two preparations of K487. The results were quantified by densitometric scanning of the RNase protection gels, and expressed as the ratio of intensities of the band arising from correct initiation of the injected keratin gene (i in Fig. 6A) and the smallest of the four protected globin bands (the intensities of the four globin bands being similar to each other). The results are tabulated in Table 1 (where experiment 6 is the one illustrated in Fig. 6A), and show that, on average, mutation of the KTF-1 binding site confers an eight-fold reduction in transcriptional activity on the keratin promoter. Comparison with the co-injected reference gene, KG487, showed that each set of embryos received, and was capable of expressing, injected DNA. DNA dot blots

Table 1. Comparison of transcriptional activity of the keratin gene, XK81A1, with (K487), and without (M157E), a functional KTF-1 binding site

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Ratio of transcription levels K487:M157E</th>
<th>Ratio of DNA levels K487:M157E</th>
<th>Transcription level relative to DNA level</th>
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<td>1.0</td>
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<td>9.5</td>
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<td>1.4</td>
<td>3.2</td>
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<tr>
<td>6</td>
<td>6.1</td>
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</tr>
<tr>
<td>Average</td>
<td>8.2</td>
<td>1.1</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Transcription and DNA levels were measured by densitometry of autoradiographs from RNase protection assays and DNA dot blots. In each experiment, transcription and DNA levels were normalised with respect to a co-injected, reference gene (see text). Experiment 6 is illustrated in Fig. 6A.
were scanned for each experiment. The ratio of transcription level to amount of template DNA is given in Table 1 (but see Discussion), and indicates that the reduced expression of M157E cannot be attributed to the injection or persistence of lower levels of M157E DNA. Thus, the binding site for KTF-1 serves to activate XK81A1 transcription, and KTF-1 is suggested to be a transcriptional activator of keratin expression in the *Xenopus* embryo.

Besides band i, representing correct initiation of the injected keratin gene, the keratin probe protects other bands specifically in injected embryos. We interpret these as representing transcription from cryptic start sites in the gene or vector, because deletion of the TATA box causes disappearance of band i, but not of these bands (data not shown). The mismatch between the RNase protection probe, which includes 5' flanking sequence, and the mutation at -157 in M157E, causes the longest of these bands to be truncated compared to its equivalent in K487-injected embryos. We observed that the additional bands are often stronger in M157E-injected embryos, as in Fig. 6A. The reason for this is not known, but reduced use of the correct initiation site in M157E may allow the cryptic start sites to be more available for transcription, for instance, if they lie within the XK81A1 transcription unit.

To investigate whether the KTF-1 site is necessary for epidermis-specific expression of XK81A1, M157E-injected embryos were dissected into epidermis and carcass (non-epidermis) fractions before RNase protection analysis (Fig. 6A(iv)). The results of four separate experiments show that although M157E expression is drastically reduced, it is still epidermis specific.

To investigate whether KTF-1 can act as an activator of promoters other than the XK81A1 gene, we tested the ability of the KTF-1 binding site to increase expression of the *Xenopus* adult β-globin gene, which is normally inefficiently transcribed on injection into embryos (Krieg and Melton, 1985; Bendig and Williams, 1984). We constructed synthetic oligos containing either the sequence of the KTF-1 binding site (oligo c6, Fig. 2B), or a sequence altered so that it did not compete for KTF-1 binding in mobility shift assays (oligo c7, Fig. 2B,C). Either c6 or c7 was cloned into plasmid 64-XβG, 500 bp upstream from the initiation site (Fig. 5). 64-XβG contains the entire *Xenopus* adult β-globin gene, including approximately 500 bp of upstream flanking sequence. The constructs were injected into *Xenopus* embryos, and transcription was analysed by Northern hybridisation (Fig. 6B). To correct for variations in RNA loading, globin expression was normalised to that of an endogenous gene, either α-actin or keratin XK81A1.

No consistent effect on transcription was observed in constructs containing only one copy of the KTF-1 binding site (data not shown). However, in 4 out of 5 experiments, constructs containing two binding sites showed much higher levels of transcription than both

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**Fig. 3.** DMS methylation interference analysis of KTF-1 binding to the keratin promoter. Binding reactions were carried out on partially methylated, end-labelled probe 1, using nuclear extracts from tailbud embryos. After separation and recovery of bound (b), and free (f) probe (see Materials and methods) the DNA was cleaved at the methylated G residues, and electrophoresed. Each band represents a G residue in the probe sequence, as shown at the left of the gel. The probe was labelled on the coding strand (+) in A, and on the non-coding strand (−) in B. Where methylation of a G residue interferes with protein binding, that residue is under-represented in the bound fraction relative to the free, as seen in the densitometric scans. Two G residues on the coding (+) strand (−155 and −154) and three on the non-coding (−) strand (−159, −160 and −161) are suppressed 2- to 3-fold in the bound fraction.
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Fig. 4. (A) Footprinting analysis of KTF-1 binding to the keratin promoter. Binding reactions were carried out on probe 1, as described for DMS interference analysis, and analysed by mobility shifting. OP-Cu ions were used to cleave the bound (b) and free (f) fractions, and the products were recovered, and electrophoresed alongside an A/G sequencing reaction of probe 1. KTF-1 binding to probe 1 gives a single footprint, covering sequences from -148 to -166 on the coding (+) strand, and -151 to -169 on the non-coding (−) strand. (B) Diagram summarising the footprinting and DMS interference analysis of KTF-1 binding to the keratin promoter. Sequence represents the XK81A1 upstream region from residue -178 to -140. '+' is the coding, and '−' the non-coding, strand. G residues shown by DMS interference to be important in KTF-1 binding are marked by arrows. The extent of the OP-Cu footprint on each strand is delimited by a square bracket. The broken line marks three residues on the non-coding strand, where KTF-1 binding results in partial blockage of OP-Cu digestion. The asterisk marks an OP-Cu hypersensitive site in the coding strand, apparently created by KTF-1 binding (Fig. 4A).

Fig. 5. Gene constructs used in functional analysis of KTF-1 binding. Solid lines represent eukaryotic DNA. Broken lines are vector sequence. Stippled boxes are XK81A1 exons. Cross-hatched boxes are human β-globin exons. Solid boxes are Xenopus β-globin exons. The restriction site in the 3′ flanking DNA, used to linearise each construct before injection, is shown: H, HindIII; K, KpnI. K487/M157E: K487 contains the coding sequence of the XK81A1 keratin gene, with 487 bp of upstream sequence, cloned into pUC18. The gene is modified by insertion of 36 bp into the first exon (marked by a vertical line), which allows detection of transcripts by RNase protection (Jonas et al. 1989). M157E is identical to K487, except that 7 bp of the promoter sequence, within the KTF-1 binding site, were altered to give an Eco RV binding site, as shown. This sequence does not compete for KTF-1 binding (see Fig. 2). The position of the mutation is marked by a vertical arrow. KG487: Fusion of XK81A1 upstream region from residue −487 to +26 (upstream promoter sequence and 5′ untranslated sequence of the first exon), to the human β globin gene at the translation initiation codon; vector is pBS+. 64-XβG: The Xenopus β-globin gene, with approximately 500 bp of upstream sequence, cloned into pSP64. Derivatives of 64-XβG, containing either functional or mutated, non-functional KTF-1 binding sites cloned into the 5′ Eco RI site at −500, are shown below. XβG-K2 contains 2 copies of oligo c6 (wild-type KTF-1 site, see Fig. 2), both in the correct orientation. XβG-M2 contains 2 copies of oligo c7 (mutant KTF-1 site, see Fig. 2), one in the correct orientation, and one in the incorrect orientation.

Thus it appears that the KTF-1 binding site can act as a strong enhancer of Xenopus β-globin transcription. However, the requirement for two copies of the site may be due to some aspect of its environment which is less favourable in the globin promoter than in XK81A1. Dissection of the injected embryos shows that the KTF-1 site enhances globin expression in both epidermal and non-epidermal tissues (Fig. 6B(iii)).
Fig. 6. Functional analysis of KTF-1 binding. (A) Mutation of the KTF-1 binding site in the keratin promoter. (i): Agarose gel showing DNA preparations for embryo injection, illustrating that similar concentrations of each construct were injected. Upper bands: K, K487 (XK81A1 gene with wild-type KTF-1 binding site); M, M157E (XK81A1 gene with mutated KTF-1 binding site). Lower band: KG487 (reference gene) in both lanes. Each DNA preparation was injected into fertilised Xenopus embryos, and expression from the injected genes analysed at tail-bud stage. (ii) and (iii): DNA dot blot and RNase protection analysis of injected embryos. Each dot, or each lane, contains DNA or total RNA from the equivalent of one embryo. The probes were specific for keratin XK81A1 (ii), or human β-globin (iii). Dot blots show the amount of injected DNA remaining at tailbud stage. e, RNA from endogenous keratin gene; i, RNA from injected keratin or globin gene; C, control, uninjected embryos; M, M157E/KG487 injected embryos; K, K487/KG487 injected embryos. The samples analysed in (ii) and (iii) are from the same experiment. M157E-injected embryos express keratin from the injected gene to a significantly lower level than K487-injected embryos, even though they contain similar amounts of injected DNA, and express the reference injected gene, KG487, to a similar extent. Longer protected bands, presumably representing transcription from upstream cryptic start sites in the injected plasmid, are also equally strong in M157E- and K487-injected embryos. (iv): Epidermal specificity of M157E expression. M157E-injected embryos were dissected into epidermal (Ep) and carcass (Ca) fractions, and subjected to DNA and RNA analysis. Each lane contains material from the equivalent of one embryo. Most of the injected DNA is in the larger, carcass fraction, but keratin expression from both the injected and the endogenous gene is mainly in the epidermis. (B) KTF-1 binding can confer high levels of expression on a heterologous promoter. (i): Agarose gel showing DNA preparations for injection. X, 64-XβG; K2, XβG-K2 (two wild-type KTF-1 binding sites); M2, XβG-M2 (two mutated KTF-1 binding sites). DNA was injected into fertilised Xenopus embryos, and expression analysed at the tailbud stage. (ii): DNA dot blot and RNA Northern blot analysis of injected embryos. Dot blots were hybridised with Xenopus β-globin specific probe, to show the amount of injected DNA remaining. Duplicate RNA gels were hybridised with probes specific for Xenopus β-globin (injected, i), and for keratin XK81A1 and α-actin (endogenous, e) to allow standardisation. Each dot, or each lane, contains DNA or total RNA from the equivalent of one embryo. C, uninjected, control embryos. XβG-K2 injected embryos (K2) express globin from the injected gene to a higher level than either 64-XβG injected embryos (X), or XβG-M2 injected embryos (M2), containing similar amounts of injected DNA. (iii): KTF-1 binding does not confer epidermal specificity on a heterologous promoter. XβG-K2 injected embryos were dissected into epidermal (Ep) and carcass (Ca) fractions, and subjected to DNA and RNA analysis. Each lane contains material from the equivalent of one embryo. Hybridisation of a duplicate RNA gel with probes for the endogenous transcripts keratin XK81A1 (epidermis specific), and α-actin (mesoderm specific), shows purity of the dissection. Globin transcripts from the injected gene are detected in both tissue fractions, reflecting the distribution of the injected DNA.

Discussion

In this paper, we identify a protein-binding sequence in the promoter of the Xenopus keratin gene XK81A1, and demonstrate the role of this site in the control of embryonic keratin expression. We show that the site binds a nuclear protein from Xenopus embryos, KTF-1, in vitro, and that its presence in the XK81A1 promoter strongly enhances expression of this gene in embryonic epidermis. It is, therefore, likely that KTF-1 functions
Transcription levels were corrected for differences in RNA loading by comparison with signal from an endogenous gene in each sample. Transcription and DNA levels of the test plasmids, which by comparison with signal from an endogenous gene in each experiment, are expressed as a ratio with respect to 64-XqG, which was injected into sibling embryos as a standard in each experiment. Experiment 3 is illustrated in Fig. 6B.

Table 2. Effect of the addition of functional or nonfunctional KTF-1 binding sites on transcriptional activity of the Xenopus β-globin gene

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Transcription and DNA levels were measured by densitometry of autoradiographs from Northern blots and DNA dot blots. Transcription levels were corrected for differences in RNA loading by comparison with signal from an endogenous gene in each sample. Transcription and DNA levels of the test plasmids, which have either functional (XqG-K2) or non-functional (XqG-M2) KTF-1 binding sites, are expressed as a ratio with respect to 64-XqG, which was injected into sibling embryos as a standard in each experiment. Experiment 3 is illustrated in Fig. 6B.

in the embryo as a transcriptional activator of keratin expression, although we cannot formally exclude the possibility that another protein, with similar DNA-binding properties, acts at the KTF-1 site in vivo.

As shown in Tables 1 and 2, there was some variability between experiments in the level of transcriptional activation conferred by a functional KTF-1 site or sites, especially when the sites are transferred to a heterologous promoter (Table 2). Some of the variability may be attributed to differences in the level of plasmid DNA persisting at the time of analysis; however, since we do not know what fraction of injected DNA is transcribed, and whether any of the measured expression arises from transiently high levels of plasmid DNA, it may not be strictly valid to express transcription relative to the amount of template DNA. Another explanation might be heterogeneity between clutches of embryos, in the level of KTF-1.

Although the absence of a functional KTF-1 binding site reduces expression of XK81A1, it does not alter its tissue specificity (at least within the limits of our assay). Similarly, insertion of KTF-1 binding sites increases transcription from a heterologous promoter but does not confer epidermal specificity. Thus, it appears that KTF-1 may be an example of a more general transcription factor contributing to the expression of a tissue-specific gene. Consistent with this interpretation, nuclear extracts made from either epidermal or carcass fractions of tailbud embryos were found to contain KTF-1 activity (assayed by DNA mobility shift), although KTF-1 appeared to be especially abundant in epidermis (data not shown). Dynan (1989) suggests that such non-specific transcription factors, which are frequently found to bind the promoters of regulated genes, may create an environment where transcription is highly responsive to inducible or tissue-specific factors.

The KTF-1 binding site may act in concert with a number of other regulatory elements to give epidermis-specific keratin expression. Many transcriptional control regions have a modular structure, which may include some degree of redundancy. For instance, Herr and Clarke (1986) have shown that a functional SV40 enhancer can be built either by duplication of homologous sequence motifs, or by combinations of heterologous pairs of motifs. In the keratin promoter, there might be multiple control elements, including the KTF-1 site, combinations of which would give tissue-specific expression. The results of deleting the XK81A1 promoter support such a model; progressive 5'→3' deletions from -487 to -100 show gradual loss of tissue specificity (Jonas et al. 1989), but a series of individual, internal 50 bp deletions covering the same region, in a background of 1310 bp of upstream sequence, were all tissue specific (E. Jonas, unpublished observations). Similar results have been obtained by Mohun et al. (1989), working on another tissue-specific Xenopus embryonic gene, cardiac actin. Here, deletion of a single protein-binding motif, CArG box 1, from the promoter, abolishes expression of the injected gene, but transfer of one or more copies of the CArG box fails to confer muscle-specific expression on a heterologous promoter. This implies that the actin promoter must contain other control elements, besides the CArG box, but no evidence of them can be found by mutation of small regions of the promoter.

Keratin expression in Xenopus laevis is both spatially and temporally regulated. Different sets of keratin genes are expressed in eggs, embryos and adult tissues (Franz et al. 1985; Jamrich et al. 1987; Ellison et al. 1985). XK81A1 is one of a number of Xenopus type I keratins, including other members of the XK81 family (Miyatani et al. 1986) and the distantly related type I gene, XK70A (Winkles et al. 1985), which show similar but not identical expression patterns in the early embryo. Upstream sequence information is available for two other XK81 genes, XK81B1 and XK81B2 (Miyatani et al. 1986), and for XK70A (Krasner et al. 1988). No exact match to the XK81A1 KTF-1 site is found in any of these genes. The closest match is in XK81B2, which contains the sequence GCCCTGAAGGT (reading 3'→5') 180 bp upstream of the initiation site. This matches the KTF-1 binding site at 8 out of 11 nucleotides, but no protein-binding data are yet available for the XK81B2 gene. There is also some sequence similarity between the KTF-1 site and an 8 bp element (consensus GCCGTGPG, previously noted as laying between -250 and -270 in the three sequenced XK81 genes, including 81A1 and XK70A (Jonas et al. 1989), and having the same sequence as the recognition element for AP-2, a cell-type-specific transcription factor of humans (Williams et al. 1988). However, we have so far been unable to detect any nuclear factor binding to this site in the XK81A1 promoter, so it is
unclear whether it plays a role in the gene's regulation, and its relationship, if any, to KTF-1 remains unknown. Thus the question of whether KTF-1 also regulates other embryonic keratins or is specific for XK81A1 remains open.

There is some information about sequences regulating keratin gene expression in organisms other than *Xenopus* (see the review by Steinert and Roop, 1988). Blessing et al. (1989) identified a 5′ upstream region, between nucleotides −180 and −605, of the bovine cytokeratin gene, CKIV, which shows tissue-specific enhancer activity. We note that this region contains the sequence ACCCCTAGAGA, which matches the KTF-1 binding site in 8 out of 11 nucleotides. In addition, a human 50×10^3 M₁ type I epidermal cytokeratin gene (Marchuk et al. 1985) contains the sequence ACCCAGGCT (3′→5′) at approximately −100, which matches the KTF-1 site in 9 out of 11 nucleotides.

Apart from its limited similarity to the potential AP-2 site, we have not found homology of the KTF-1 binding site to that of any published transcription factor. Thus KTF-1 may represent a previously unknown transcriptional activator. However, given the extreme degeneracy of binding sequences for many transcription factors (see Johnson and McKnight, 1989), for review this issue may only be resolved by purification and/or cloning of KTF-1.

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