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The DNA-binding protein E12 co-operates with XMyoD in the activation of muscle-specific gene expression in *Xenopus* embryos

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Two alternatively spliced products of the human E2A gene, E12 and E47, encode helix–loop–helix DNA-binding proteins. Here we describe the isolation of two *Xenopus* cDNAs; one, XE12, is structurally similar to human E12 and the other contains a sequence similar to E47. Transcripts of both cDNAs are present at all the stages of *Xenopus* development tested and in all regions of the embryo. The DNA binding properties of *in vitro* translated XE12 are indistinguishable from those of human E12. We have shown previously that an embryonic muscle DNA-binding activity, EMF1, that binds to a promoter sequence required for the expression of the cardiac actin gene, contains the *Xenopus* myogenic factor XMyoD. Here we show that it also contains protein that interacts with an anti-E12 antiserum, suggesting that XE12 and XMyoD proteins, or very similar ones, are present in EMF1. We have addressed the functional role of XE12 in muscle gene transcription in *Xenopus* embryos by injecting *in vitro* synthesized RNA into the two cell embryo. Overexpression of XE12 and XMyoD augments by >10-fold the ectopic activation of the endogenous cardiac actin gene that can be produced by XMyoD alone. Our DNA binding results strongly suggest that this effect is mediated through a direct interaction of the XE12–XMyoD complex with specific sites in the cardiac actin promoter. We suggest that XE12 is functionally important in muscle gene activation in embryonic development.

Key words: helix–loop–helix proteins/muscle/myogenesis/*Xenopus*

Introduction

A number of helix–loop–helix DNA-binding proteins share similarities with the proteins encoded by the human E2A gene (Murre *et al.*, 1989a; Henthorn *et al.*, 1990a; Nelson *et al.*, 1990). The original description of E2A proteins was in cells associated with the lymphoid system, but these proteins have now been shown to be present in a wide variety of tissues and in several species. In humans, the E2A gene produces two transcripts, E12 and E47, that differ by the use of an alternative exon (Murre *et al.*, 1989a; Sun and Baltimore, 1991), while a transcript E2-2, which is almost indistinguishable from E12, is encoded by a separate gene (Henthorn *et al.*, 1990a). The proteins from these genes have been implicated in the transcriptional regulation of other genes in several situations such as the immune system (Henthorn *et al.*, 1990a), the endocrine and exocrine pancreas (Nelson *et al.*, 1990) and in muscle, which has been extensively studied (see review by Weintraub *et al.*, 1991).

The products of the E2A genes can form complexes with certain tissue-specifc proteins that contain a helix–loop–helix domain. In the case of muscle, the myogenic factors MyoD, myogenin, Myf5 and MRF4/herculin/Myf6 have all been shown to interact with E2A proteins via the helix–loop–helix domain (Murre *et al.*, 1989b; Brennan and Olson, 1990; Chakraborty *et al.*, 1991). The complexes produced by these interactions bind specifically to the DNA-binding site, CANNTG, called the E-box. The formation of a protein complex *in vitro* between an E2A-like protein and a tissue-specific factor greatly increases the binding affinity of the complex for the E-box. This sequence is present in several muscle-specific promoters and enhancers including those of the *Xenopus* (Taylor *et al.*, 1991) and human (Sartorelli *et al.*, 1990) α-cardiac actin gene, the acetylcholine receptor (Piette *et al.*, 1990), the muscle creatine kinase (MCK) gene (Lassar *et al.*, 1989; Braun *et al.*, 1990; Brennan and Olson, 1990), the myosin light chain 1/3 gene (Wentworth *et al.*, 1991) and the troponin I gene (Lin *et al.*, 1991).

Work in *Xenopus* has shown that high level tissue-specifc expression of the *Xenopus* cardiac actin gene is dependent upon two regions of the promoter. One of these, called the M-region, contains three E-box motifs and is able to bind *Xenopus* MyoD (XMyoD) (Taylor *et al.*, 1991). This region of the promoter also binds a sequence-specific DNA-binding activity found in embryonic muscle called EMF1, that contains XMyoD complexed with other proteins. In this paper, we investigate the idea that possible partners for XMyoD in the *Xenopus* embryo are the *Xenopus* equivalents of the E2A gene products. This idea is supported by observations made on muscle cell-line extracts, which have been shown to contain protein complexes that cross-react with both anti-XMyoD and anti-E12 antisera and bind to an E-box sequence (Murre *et al.*, 1991).

We address two further key questions in this study. First, do the E2A gene products have a role in the activation of muscle gene expression in the developing embryo? Secondly, is this function mediated by interaction with XMyoD? Previously we have shown, by injecting XMyoD RNA into the two cell embryo, that XMyoD can activate transcription of the *Xenopus* cardiac actin gene (Hopwood and Gurdon, 1990). This technique provides a bridge between the transfection experiments in cultured cells and the normal events of myogenesis. In the work presented here we extend our observations by investigating the role of a *Xenopus* cDNA, XE12, that is structurally and functionally similar to human E12. We show not only that the RNA from this cDNA is able to co-operate with XMyoD to activate the endogenous cardiac actin gene, but also that XE12 can augment the effect of XMyoD alone by >10-fold. We

provide evidence that this effect is through direct binding of an XE12−XMyoD-containing complex found in embryonic muscle to the cardiac actin promoter. Together these experiments suggest that XE12 and XMyoD co-operate to activate the endogenous cardiac actin gene directly in *Xenopus* embryonic development.

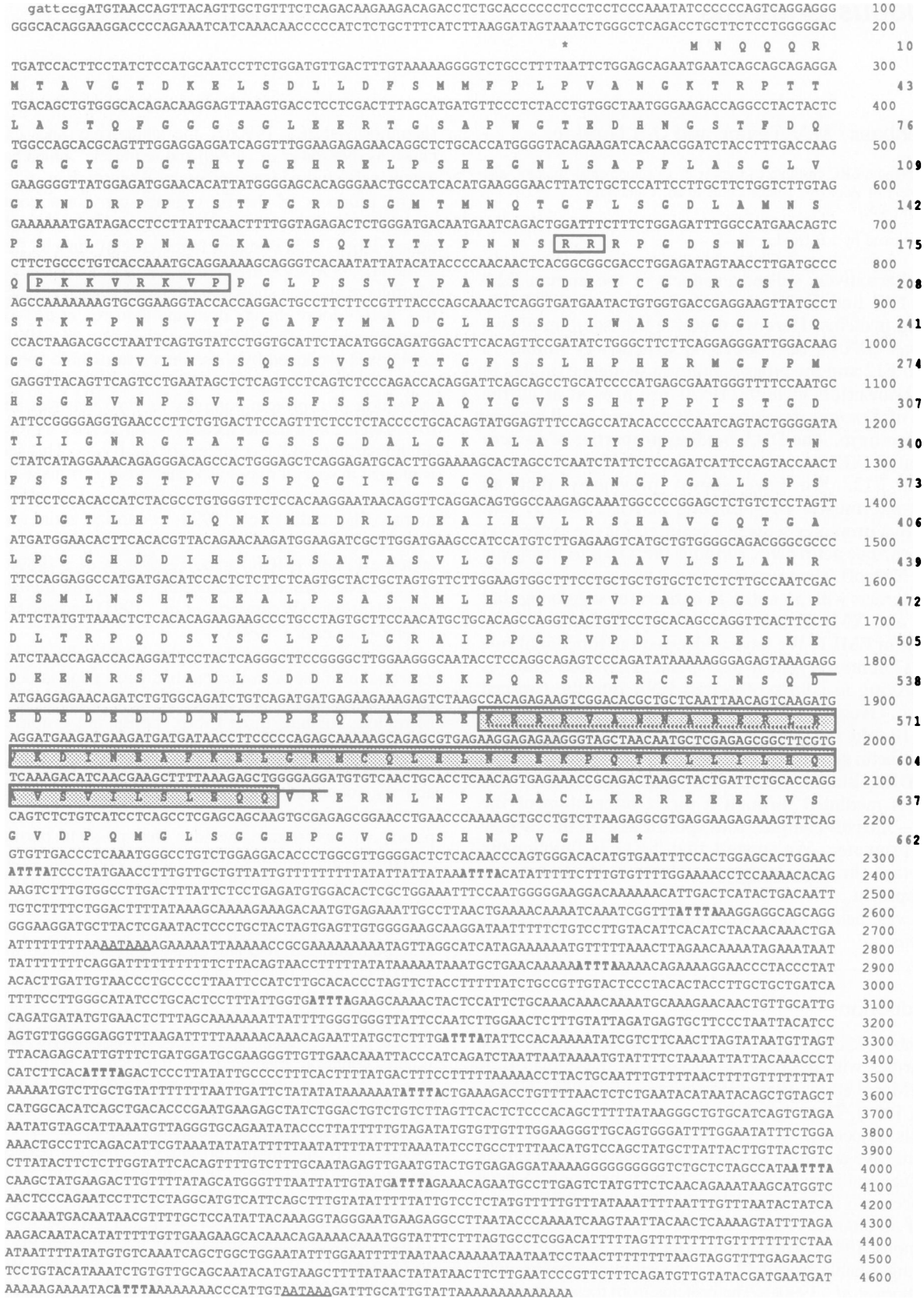


Fig. 1. The cDNA sequence of XE12. The region of the putative nuclear localization signal is boxed; the region of the alternative exon found in E47 is overlined, and the basic and helix−loop−helix domains are shaded. The numerous ATTTA sequences in the 3′-untranslated region are in bold type. The two polyadenylation signals are underlined.

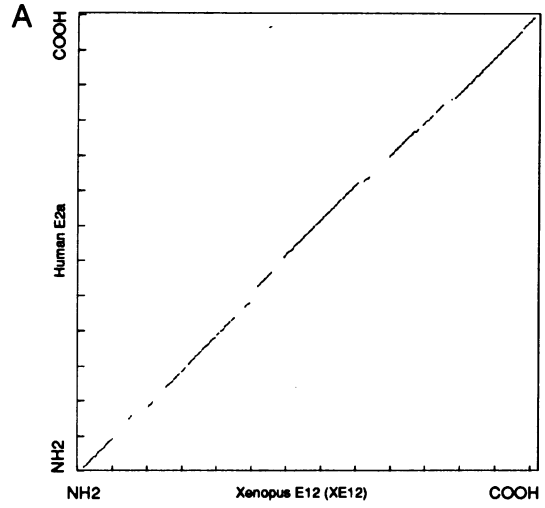
Results

A Xenopus cDNA is a homologue of human E12

We screened a *Xenopus laevis* neurula (stage 17) cDNA library (Kintner and Melton, 1987) with probes from the human cDNA clones E12 and E47 (Murre *et al.*, 1989a). Purified positive cDNAs all hybridized to each other at high stringency. Two cDNAs (XE12.7 and XE12.15) were of a length similar to the transcript size subsequently estimated by Northern blots (see below), and one of these, XE12.7, was chosen for sequencing. Examination of the sequence at the putative 3′-end suggested that XE12.7 was probably not full-length. Double-stranded sequencing of the ends of XE12.15 showed the putative 5′-end to be indistinguishable from XE12.7, while the other end extended the sequence of XE12.7 by a further 43 bases, which included a polyadenylation signal 15 bases upstream of a poly(A) tract of 13 As. Although these cDNA clones probably arise from transcripts of the same gene, we have not formally excluded the possibility that they originate from transcripts of different genes.

Figure 1 shows the composite cDNA of XE12.7 and XE12.15 and their conceptual translation. The full length transcript based on the sequence is 4.6 kb in length, a size that is confirmed by Northern analysis of RNA extracted from embryos. There is a single long open reading frame of 1989 bases coding for 663 amino acids, similar to the human clone which encodes 654 amino acids (Kamps *et al.*, 1990). The first in-frame methionine is preceded by an in-frame stop codon. The conceptual translation of clone XE12.7/15 has extensive homology with human E2A.E12 (Kamps *et al.*, 1990) and other E2A-like transcription factors (Figure 2A), the region of greatest similarity being the helix−loop−helix domain (Figure 2B). This domain is the same as human E12 with the exception of two residues, a Lys_{Xen} for an Arg_{Hum} and a Ser_{Xen} for an Asp_{Hum}. The XE12 amino acid sequence in this region is more similar to human E12 than to the E2A gene product E47 (see below). In common with other E2A-like transcription factors, a small conserved region in the amino-terminus of the protein contains a bipartite nuclear localization signal (Robbins *et al.*, 1990), with a sequence almost indistinguishable from that found in the SV40 T antigen (PKKKRKV) (Kalderon *et al.*, 1984; Miller *et al.*, 1991).

The *Xenopus* cDNA clone XE12.7.15 that we have isolated has a 3′-untranslated region of 2.3 kb. This is also true of the human cDNA encoding E2A.E12. Both the *Xenopus* and human E12 sequences have two polydenylation signals within this region, the internal one in the *Xenopus* clone being at position 2712. One striking difference between the 3′-untranslated regions of the *Xenopus* and human clones is the presence within the *Xenopus* cDNAs of numerous ATTTA sequences, which are absent from the human clone. A related human HLH transcription factor, SCL, has been found to contain several of these sequences (Aplan *et al.*, 1990) that are associated with message instability in other genes (Shaw and Kamen, 1986; Shyu *et al.*, 1989, 1991). On the basis of the amino acid similarity between the *Xenopus* cDNA clone XE12.7.15 and the human cDNA E2A.E12, we believe this cDNA to be the *Xenopus* homologue. We have called this cDNA XE12.



B

			Basic	Helix	Loop	Helix
1.	Xenopus	E12	KERRVANNARERLRVKDINEAFKELGRMCQ----LHLN----------SEKPQTKLLILHQAVSVILNLEQQ			
2.	human	E12	KERRVANNARERLRVRDINEAFKELGRMCQ----LHLN----------SEKPQTKLLILHQAVSVILNLEQQ			
3.	mouse	E12	KERRVANNARERLRVRDINEAFKELGRMCQ----LHLS----------SEKPQTKLLILHQAVAVILSLEQQ			
4.	rat	Pan2	KERRVANNARERLRVRDINEAFKELGRMCQ----LHLS----------TEKPQTKLLILHQAVAVILSLEQQ			
5.	human	E2-2	KERRMANNARERLRVRDINEAFKELGRMVQ----LHLK----------SDKPQTKLLILHQAVAVILSLEQQ			
6.	dog	TEF	KERRMANNARERLRVRDINEAFKELGRMVQ----LHLK----------SDKPQTKLLILHQAVAVILSLEQQ			
7.	Xenopus	E47	RERRMSNNARERVRVRDISEAFKELGRMVQ----MSNK----------ADKAQTKLIILQQAVAVIMSLEEQ			
8.	human	E47	RERRMANNARERVRVRDINEAFRELGRMCQ----MHLK----------SDKAQTKLLILQQAVQVILGLEQQ			
9.	mouse	E47	RERRMANNARERVRVRDINEAFRELGRMCQ----LHLK----------SDRAQTKLLILQQAVQVILGLEQQ			
10.	rat	Pan1	RERRMANNARERVRVRDINEAFRELGRMCQ----LHLK----------SDKAQTKLLILQQAVQVILGLEQQ			
11.	human	E2-5	RERRMANNARERVRVRDINEAFRELGRMCQ----MHLK----------SDKAQTKLLILQQAVQVILGLEQQ			
12.	Drosphila	da	KERRQANNARERIRIRDINEALKELGRMCM----THLK----------SDKPQTKLGILNMAVEVIMTLEQQ			

Fig. 2. (**A**) A dot-plot showing the similarity between the conceptual translations of XE12 and human E12.E2A (Kamps *et al.*, 1990). (**B**) An alignment of the conceptual translations of several cDNA clones in the region of the helix−loop−helix domains. Sources of sequences: 2, Murre *et al.* (1989a); 3, Benezra *et al.* (1990); 4, Nelson *et al.* (1990); 5, Henthorn *et al.* (1990b); 6, Javaux *et al.* (1991); 8, Murre *et al.* (1989a); 9, Benezra *et al.* (1990); 10, Nelson *et al.* (1990); 11, Henthorn *et al.* (1990); 12, Caudy *et al.* (1988);

An alternatively spliced cDNA resembling human E47 is also present in Xenopus

We wished to know whether *Xenopus* also contains cDNAs with a sequence more similar to that found in human E47 than E12. The sequence responsible for the DNA-binding differences between the human E12 and E47 clones is found in the A-box, a stretch of 18 amino acids just amino-terminal to the basic region (Chakraborty *et al.*, 1991; Sun and Baltimore, 1991). We reasoned that should the same sequence differences be present in *Xenopus* transcripts, then the region we had assumed in XE12 to be the A-box (Figure 3A) would be most different in a clone resembling human E47. We therefore synthesized an oligonucleotide complementary to a portion of the A-box of XE12, and used this to identify which of the originally isolated λ clones failed to cross-hybridize. Two of the 19 isolated clones failed to cross-hybridize; the region of these that included the basic helix−loop−helix domain was identified by hybridization, subcloned and sequenced. Translation of this region revealed a single open reading frame with an amino acid sequence more similar to human E47 than to either *Xenopus* E12 or human E12 (Figure 3B). We refer to these cDNAs as XE47. When XE47 is compared with XE12, the greatest divergence of amino acid sequence is seen in a 231 nucleotide stretch encoding 77 amino acids. On each side of this sequence the nucleotide compositions of XE12 and XE47 are the same. These features are compatible with alternative exon use producing a difference in the basic helix−loop−helix and A-box of the XE12 and XE47 proteins.

XE2A transcripts are present at a constant level during early development

We used Northern blot analysis to determine the size and relative abundance of transcripts from the XE2A gene during early development (Figure 4). Using a probe that detects both XE12 and XE47, two transcripts of ~4.6 and 4.0 kb are seen and these migrate on either side of the 28S RNA. To exclude the possibility that the lower transcript was the result of degraded RNA displaced by the large amount of 28S RNA, poly(A)$^+$ RNA from stage 23 (early tailbud) was analysed. Two prominent bands of equal intensity were readily detected. Analysis of the Northern blots with probes made from different regions of the cDNA failed to distinguish between the two transcripts (data not shown). The nature of these duplicate transcripts is unclear. One possible explanation is that they are the result of other alternative

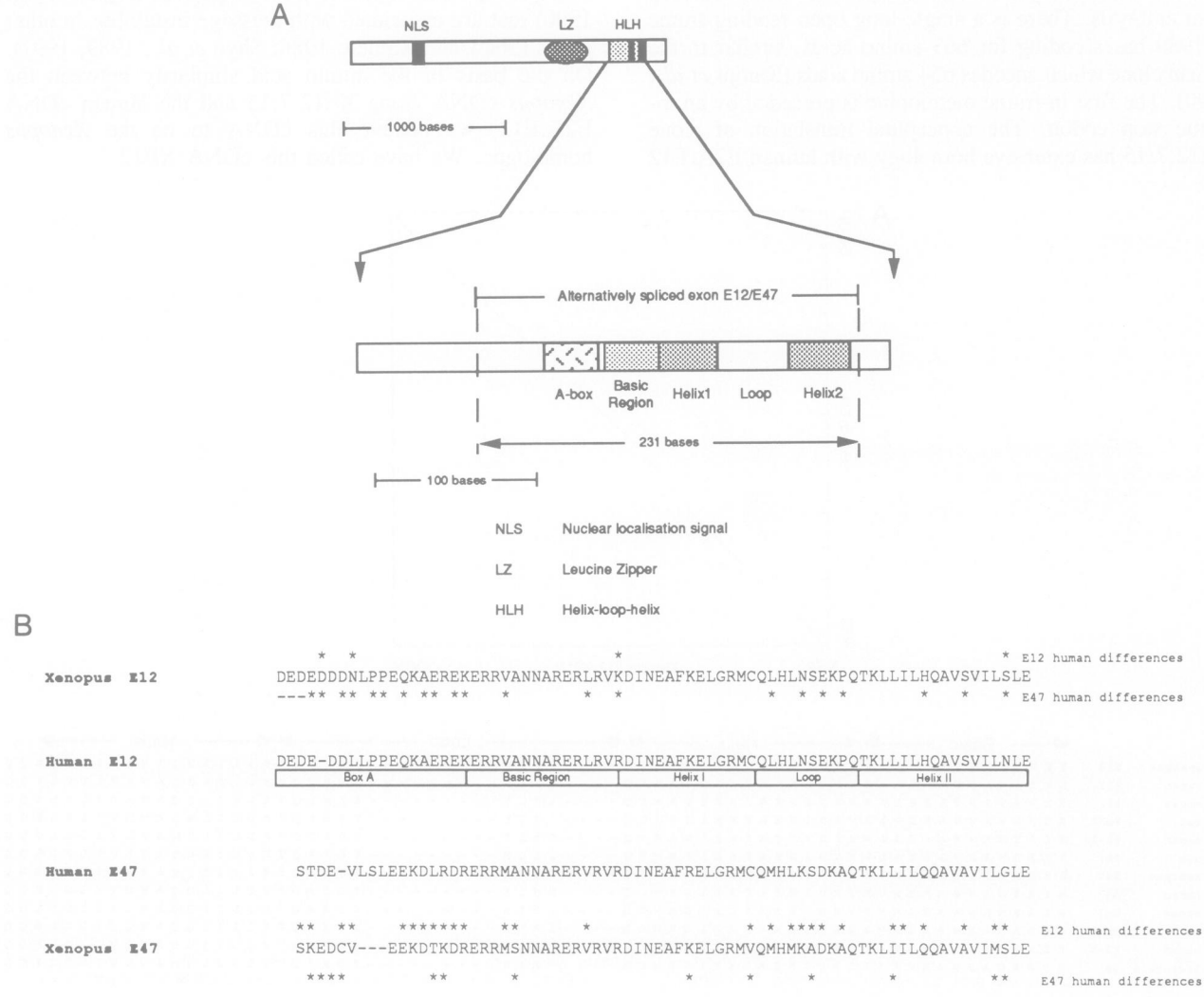


Fig. 3. (**A**) A diagram of the coding region of the XE12 cDNA showing regions with a designated function. (**B**) An alignment of the conceptual translation of the human E12 and E47 cDNAs and their *Xenopus* equivalents in the regions of the A-box, basic and helix−loop−helix domains. The difference in the amino acids between the *Xenopus* and human clones is highlighted by asterisks above and below the sequence.

splicing events that we have not detected. Another explanation is that they represent transcripts from the non-allelic copies found in the pseudo-tetraploid genome of *Xenopus laevis* (Kobel and Du Pasquier, 1986). They are not the result of termination at the internal poly(A) addition site since transcripts of this size would be much smaller and none were detected.

The number of transcripts remains approximately constant during early development, in contrast to cardiac actin transcripts for example, which can first be detected at the mid-gastrula, and which accumulate rapidly during the whole period of early development (Mohun *et al.*, 1984). The lower band appears much less abundant than the upper when total RNA is used, but not when poly(A)$^+$ RNA is used. This effect may be due to the large amount of 28S RNA which might obscure the signal from the lower transcript more than the upper.

The relative abundance of XE12 and XE47 transcripts remains constant during the early development of the embryo

It is important to know whether the XE12 and XE47 transcripts are differentially regulated either temporally during early development or spatially in the embryo. We have used RNase protection assays with a probe spanning the region of the alternative exon of XE12 to determine the relative abundance of XE12 and XE47 transcripts. The results are shown in Figure 5. XE47 transcripts are more abundant than XE12 transcripts from the oocyte to tailbud stage, while the ratio of the XE12 to XE47 transcripts remains constant at all these stages. Maternal transcripts of both types are seen in oocytes and cleavage embryos, but in subsequent stages from blastula to tadpole there is little change in the transcript number and the level remains approximately constant.

We used dissected material of regions from late neurula embryos (stage 19) to examine the spatial distribution of these two types of transcript. Protection assays using the probe described above show that transcripts of both XE12 and XE47 are present in all regions of the embryo and that the relative abundance of each is maintained between different regions (data not shown). There is no preferential expression in muscle or somite. Both the temporal and spatial distribution of XE12 and XE47 transcripts are compatible with the expression of the E2A-like genes in other systems (Murre *et al.*, 1989a; Henthorn *et al.*, 1990a; Kamps *et al.*, 1990). Any regulation of XE12 and XE47 that may exist in *Xenopus* embryos does not seem to be either by the selective use of this alternative sequence in the HLH domain or by the regulation of transcript number.

XE12 has the same DNA binding properties as human E12

We have chosen to concentrate on the DNA-binding properties of XE12. As a first test of the function of the XE12 clone we carried out a series of DNA-binding experiments using *in vitro* synthesized XE12 protein and a DNA probe from the cardiac actin promoter. This region of the cardiac

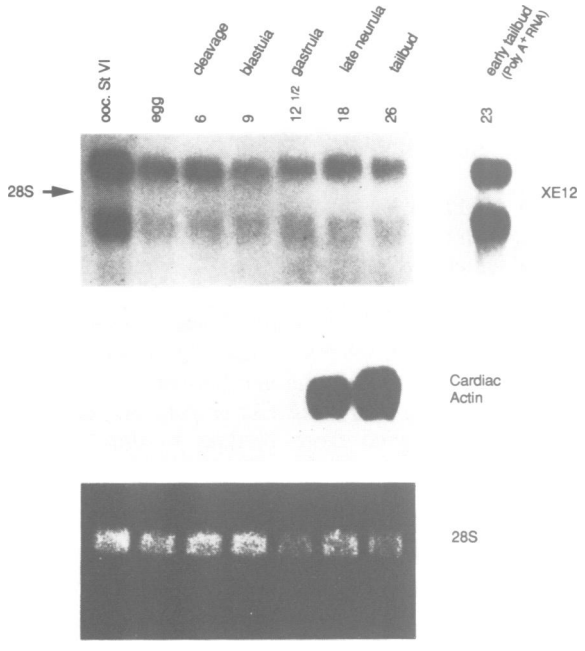


Fig. 4. A Northern analysis of developmentally staged total RNA (stage numbers indicated) and poly(A)$^+$ RNA from stage 23 embryos. Each lane was loaded with 10 μg of total RNA or with 50 ng of poly(A)$^+$ RNA. The blot was stripped and reprobed with a cardiac actin-specific probe (Mohun *et al.*, 1984) for comparison (middle panel). Ethidium bromide staining of the 18S RNA (lower panel) shows approximately equal loading.

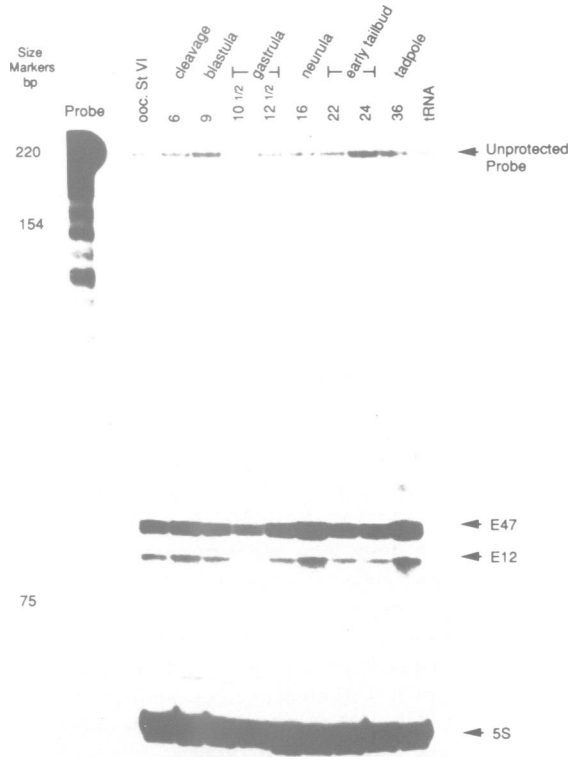


Fig. 5. An RNase protection assay of developmentally staged RNA to distinguish transcripts containing the E12 or E47-like sequence in the region of the helix−loop−helix domain. Each lane contains the total RNA extracted from one embryo or oocyte. The lower panel shows a protection assay performed on the 5S RNA in the same sample to indicate the amount of RNA extracted.

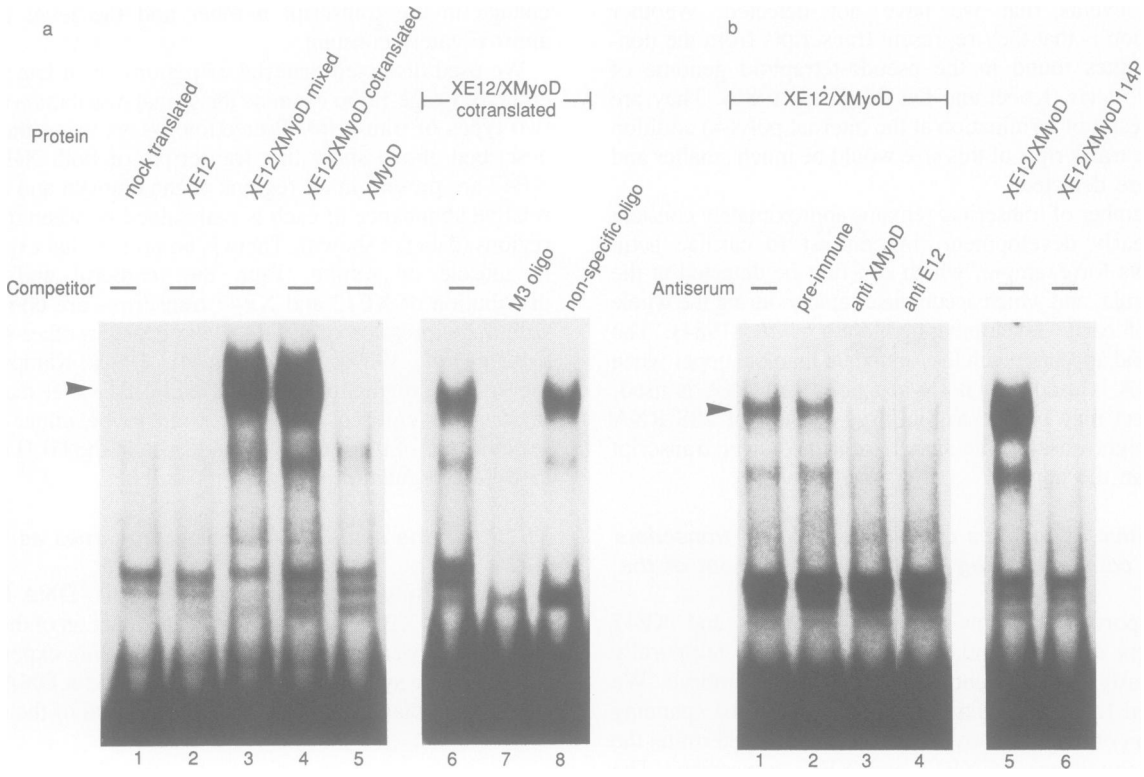


Fig. 6. (**A**) *In vitro* translated XE12 and XMyoD produce a DNA-binding complex that recognizes the M-region of the cardiac actin promoter. An electophoretic mobility shift assay (EMSA) is shown with *in vitro* translated protein. (1) mock translated reticulocyte lysate; (2) XE12 translated alone; (3) XE12 and XMyoD translated separately; (4) XE12 and XMyoD co-translated; (5) XMyoD translated alone. The specificity of the complex (arrow) is shown by competition with specific M3 oligonucleotide (100 ng) but not with 100 ng of a non-specific oligonucleotide (lanes 6–8). (**B**) XE12 and XMyoD co-translated *in vitro* produce a protein complex (arrow) that is recognized by either an XMyoD antiserum or a human E12 antiserum. (1) no antiserum; (2) pre-immune rabbit serum; (3) XMyoD antiserum; (4) human E12 antiserum. The XE12–XMyoD protein complex is dependent upon the helix–loop–helix domain. Lanes 5 and 6 show the effect of using XMyoD with a point mutation in the helix–loop–helix domain (XMyoD114P).

actin promoter, which we have described previously as the M3 probe, contains three E-boxes (Taylor *et al.*, 1991). Our results show that no specific binding of XE12 alone was detected (Figure 6A, lane 2). However, XE12 mixed with XMyoD (lane 3) or co-translated (lane 4) produced an M3 DNA-binding activity, which was shown to be specific by competition with M3 oligonucleotide, but not with a non-specific oligonucleotide (lanes 6–8). XMyoD when used alone produced little binding activity (lane 5), but when used in combination with XE12 the resulting complex had a much greater binding affinity than either protein alone. The use of human E12 and XMyoD antisera to deplete the proteins before binding confirmed that it contained both proteins (Figure 6B, lanes 1–4). Finally, the formation of this complex was dependent upon an intact HLH domain of XMyoD, since there was no detectable binding when XE12 was co-translated with XMyoD114P (lanes 5 and 6), which contains a point mutation that disrupts the HLH motif (Hopwood and Gurdon, 1990). Together, these DNA-binding characteristics identify XE12 as a DNA-binding protein that is able to interact with XMyoD. The similarity of its behaviour to human E12 suggests that XE12 is indeed a functional homologue of human E12 (Murre *et al.*, 1989a,b).

XE12 protein is a component of embryonic muscle and binds to the cardiac actin promoter

We have suggested previously that EMF1, a DNA-binding activity from embryo somites that contains XMyoD, might be a complex between this protein and products of the *Xenopus* E2A gene (Taylor *et al.*, 1991). Since EMF1 binds to the functionally important M-region of the cardiac actin promoter, the proteins contained in the complex are likely to play a significant role in the activation of this gene in the embryo.

When XMyoD is mixed with extracts made from neurectoderm or from whole embryos, a protein(s) is (are) recruited which combines with XMyoD to generate a DNA-binding complex that migrates indistinguishably from EMF1 (Taylor *et al.*, 1991). This binding activity is dependent upon an intact helix–loop–helix domain. Here we show that when it is incubated with the anti-human E12 antiserum, which also recognizes *Xenopus* E12 (Figure 6B, lane 4), the complex is prevented from binding to the M3 probe (Figure 7, lanes 1–4). This supports the idea that at least one recruited protein is indeed a product of the *Xenopus* E2A gene.

Is XE12 a component of embryonic muscle EMF1? Figure 7 shows that the antiserum against human E12 prevented EMF1 binding to the M3 probe in contrast to pre-immune control serum (lanes 5–8). Similarly, an antiserum against XMyoD also prevented the EMF1 extracted from tadpole tail (a region of the embryo with a large amount of muscle) from binding, a result shown previously for embryo somites (Taylor *et al.*, 1991). Together these results suggest that EMF1 is a complex containing a product of the *Xenopus* E2A gene and XMyoD. However, we found that although the migration of tail EMF1 was very similar to that of the

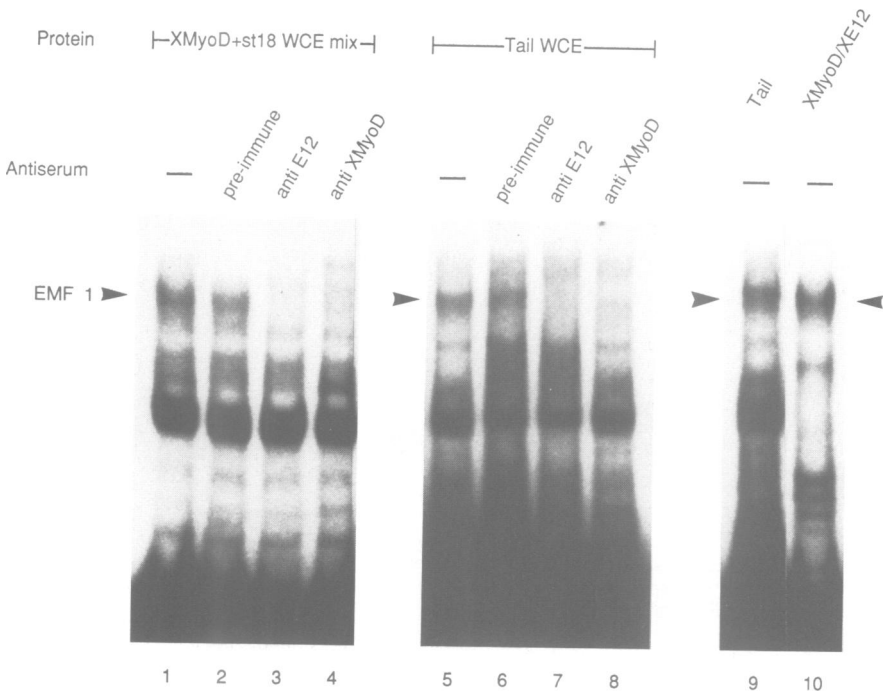


Fig. 7. XMyoD translated *in vitro* and mixed with a whole cell extract (WCE) made from stage 18 embryos recruits a protein and forms a complex (arrowed) that is recognized by the E12 antiserum (lanes 1–4). A similar binding activity is found in extracts made from the tails of stage 30–32 embryos (lanes 5–8). (1 and 5) no antiserum; (2 and 6) pre-immune rabbit serum; (3 and 7) human E12 antiserum; (4 and 8) XMyoD antiserum. The tail extract EMF1 has a mobility that is very similar to XE12 and XMyoD co-translated *in vitro* (lanes 9 and 10).

product of XE12 and XMyoD RNAs co-translated *in vitro*, it was not precisely the same (lanes 9 and 10). One explanation for this difference is that XMyoD–XE12 produced by *in vitro* translation is not modified in the same way as XMyoD–E12 in the embryo, and that this might affect the mobility of the DNA-binding complex.

The injection of XE12 augments the activation of the endogenous cardiac actin gene produced by XMyoD alone

We have described above the temporal and regional distribution of XE12 and XE47 transcripts, and addressed the binding properties of XE12 protein *in vitro*. It is, however, important to determine the function of XE12 in embryos. One method that has been used with success to address this type of question is the injection of *Xenopus* embryos at the two cell stage with *in vitro* synthesized RNA (Harvey and Melton, 1988; McMahon and Moon, 1989; Ruiz i Altaba and Melton, 1989). Experiments from this laboratory have used this procedure to investigate the effects of cloned *Xenopus* myogenic factors on muscle gene activation (Hopwood and Gurdon, 1990; Hopwood *et al.*, 1991). In these experiments we found that the injection of ~5 ng of XMyoD could activate the endogenous cardiac actin gene. However, this amount of XMyoD was not able to activate expression of proteins such as the antigen 12/101 (Kintner and Brockes, 1984) present in more differentiated muscle. More recent experiments have indicated that a dose of ~5 ng is close to a threshold above which there is a sustained activation of cardiac actin gene expression and markers such as 12/101 (N.D.Hopwood and J.B.Gurdon unpublished). We wished to know whether the co-injection of XMyoD RNA and XE12 RNA was more effective than XMyoD alone in the activation of both the cardiac actin gene and the 12/101 antigen.

The results (Figure 8) show the dramatic effect of the co-injection of XE12 and XMyoD RNA on the activation of the endogenous cardiac actin gene. The injection of XMyoD RNA alone does not detectably activate cardiac actin when 0.5 or 2 ng of RNA is injected per embryo, whereas 5 ng of XMyoD RNA produces a low level of cardiac actin transcripts. When 10 or 5 ng of XE12 RNA is co-injected into the embryos with 2 or 5 ng of injected XMyoD RNA, there is a >10-fold increase in the number of transcripts produced by the same amount of XMyoD alone. The injection of XE12 alone at a high dose (10 ng) is ineffective, as is the mutant XMyoD114P either alone (Hopwood and Gurdon, 1990) or in conjunction with XE12. When only 0.5 ng of XMyoD is injected with 10 ng of XE12, almost no actin transcripts are detected.

Furthermore, we have been able to show that the combination of XE12 and XMyoD RNAs can also activate expression of a protein associated with differentiated muscle using the antibody 12/101 (Kintner and Brockes, 1984). Histological sections of animal caps cultured from embryos injected with both XE12 and XMyoD RNA fixed at stage 31 contained 12/101 reacting cells (not shown). Embryos that had been injected with either XMyoD RNA or XE12 RNA alone showed no 12/101 positive cells.

From these results it is clear that XMyoD RNA shows a dose dependent effect on the activation of the endogenous cardiac actin gene and that co-injection of XE12 with XMyoD greatly augments the ability of XMyoD alone to activate the endogenous cardiac actin gene as well as other muscle proteins. The combination of XE12 and XMyoD results in muscle gene activation by an amount of XMyoD

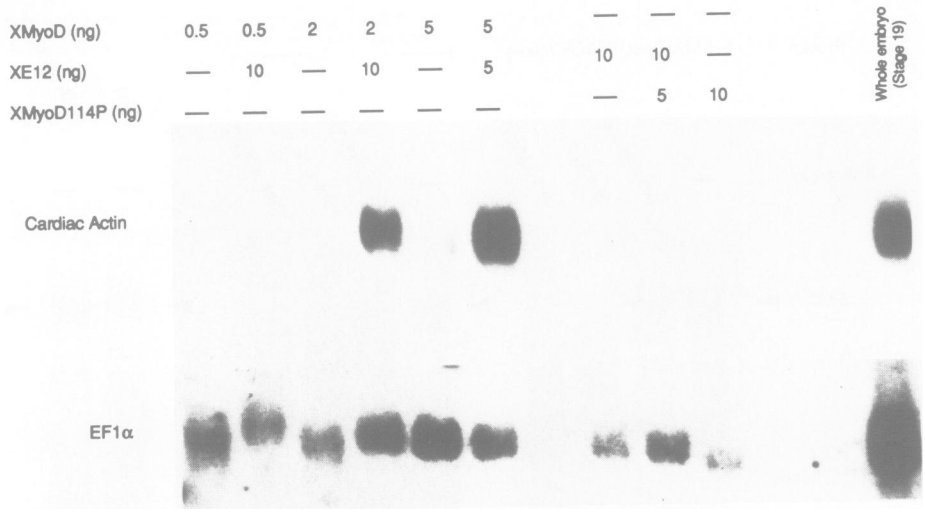


Fig. 8. A Northern analysis of animal cap explants from embryos injected with XMyoD, XMyoD114P and XE12 *in vitro* transcribed RNA. The blots were probed with the same cardiac actin-specific probe used in Figure 4. The amount of each RNA injected is shown above each lane in ng injected per embryo. Each lane contains the total RNA extracted from four animal caps frozen when sibling whole embryos reached stage 18 or from the RNA of one whole embryo at stage 18. The blot was reprobed for the transcripts of the EF1a gene (Krieg *et al.*, 1989) as an indication of the amount of RNA loaded in each lane and with a probe that recognizes injected RNAs (not shown).

more similar to that found in normal myotomal cells at the gastrula stage (Hopwood and Gurdon, 1990).

Discussion

As a prelude to investigating the function of E2A-like gene products in normal development, we have cloned and sequenced one helix−loop−helix DNA-binding protein that is the *Xenopus* homologue of the human E2A. E12 cDNA (Kamps *et al.*, 1990). A second *Xenopus* cDNA that we have isolated contains a region that shows more similarity to human E47 than human E12. The entire amino acid sequence of XE12 is highly conserved between the human and frog. This is particularly striking in the region of the alternative exon; the position of the splice being the same across the species, suggesting a similar genomic structure in this region.

The protein produced from the XE12 cDNA shares the *in vitro* binding properties of the human protein (Murre *et al.*, 1989b; Sun and Baltimore, 1991). While some workers have been able to demonstrate that E12 alone can bind to the E-box (Chakraborty *et al.*, 1991) we have not been able to show this. A possible explanation for this difference is that the other group used 200-fold more protein. If E12 binding as homo-oligomers is functionally important we have been unable to detect any effect in our over-expression assay (see below).

We have asked whether XE12 is involved in the activation of muscle gene expression in normal embryonic cells and whether this protein acts together with other proteins in this process. The co-injection of XMyoD and XE12 results in the ectopic expression of the endogenous cardiac actin gene in isolated animal caps. The number of cardiac actin transcripts produced by the injection of XE12 together with XMyoD is more than 10-fold greater than that produced by the same quantity of XMyoD alone. The addition of XE12 to XMyoD produces a similar number of cardiac actin transcripts to that seen in whole embryos, while the number produced by the same amount of XMyoD alone is barely detectable. XE12 acts with XMyoD to activate the cardiac actin gene and allows an amount of XMyoD to be injected that is closer to the level found in normal myotomal cells.

These results extend the conclusions from work performed in cultured cells. Transfection experiments into COS cells have been used to demonstrate a functional interaction between E12 and MyoD (Lassar *et al.*, 1991). However, in this experiment the target gene was an artificial promoter comprising four E-boxes coupled to a chloramphenicol acetyl transferase reporter. This type of promoter has recently been shown to give anomalous results when compared with an intact E-box-containing enhancer (Chakraborty and Olson, 1991). In contrast, the injection of RNA into *Xenopus* embryos has allowed us to test the effects of XE12 and XMyoD proteins on an unmodified and complete muscle gene promoter in embryonic cells, thereby suggesting a function for XE12 in normal development.

While we have shown an effect of XE12 and XMyoD on the cardiac actin promoter, there is also the possibility that other myogenic helix−loop−helix factors, such as XMyf-5, play a role in the embryo. XMyf-5 also forms specific DNA-binding complexes *in vitro* with XE12, a property shared by this factor in other species (Chakraborty *et al.*, 1991; Lin *et al.*, 1991). The XE12−XMyf-5 complexes formed have a higher affinity for the M-region of the cardiac actin promoter than either protein alone (our data not shown). In addition, when XMyf-5 is mixed with extracts made from embryos, it is able to recruit proteins that cross-react with the anti-human E12 antiserum, and the mobility of these complexes in an electrophoretic mobility shift assay is very similar to XMyf-5−XE12 complexes made *in vitro*. Furthermore, the ectopic expression of XMyf-5 alone produces activation of the endogenous XMyoD gene in addition to the cardiac actin gene (Hopwood *et al.*, 1991). These observations suggest that this factor may well act in conjunction with XE12 in the complex process of gene activation during myogenesis in the embryo.

We have shown here that XE12 can co-operate with XMyoD to activate the endogenous cardiac actin gene. Furthermore, we show that embryos have a tissue-specific

protein complex, EMF1, containing XE12 and XMyoD or very similar proteins. Our previous findings, from deletion analysis of the cardiac actin gene promoter (Mohun *et al.*, 1986; Taylor *et al.*, 1991), have shown that the M-region of the promoter is essential for high level tissue-specific expression in developing embryos. We suggest that the direct interaction of complexes containing XE12 and XMyoD (or very similar proteins) with this M-region regulates the transcription of the *Xenopus* cardiac actin gene in the developing embryo.

Materials and methods

cDNA library screening

A neurula stage cDNA library in λgt10 (stage 17; Kintner and Melton, 1978) was probed at low stringency with random primed fragments (Feinberg and Vogelstein, 1984) made from the whole cDNAs of human E12 and E47 (pE12 and pE47; Murre *et al.*, 1989a). The screening followed Sambrook *et al.* (1989), while the washings were as described by Hopwood *et al.* (1989a).

Positively hybridizing clones were purified by standard methods. λ DNA was analysed by Southern blotting and subcloned into plasmid (pBluescript SK, Stratagene) and M13 vectors for restriction mapping sequencing and propagating.

Limited digestion by exonuclease III (Henikoff, 1984) was used to generate nested deletions. Templates were then sequenced using the dideoxy chain termination method as described in Sequenase version 2 protocol (USB). Sequences were compiled using the DB programs of Staden (1982). Each nucleotide was sequenced at least twice on each strand.

Constructs for injection and in vitro translation.

To improve the efficiency of translation, the coding sequence and 3′UTR to the *Spe*I site at 2607 was transferred to pSP64-Xβm (Krieg and Melton, 1984). The first in-frame methionine codon at position 280 on XE12 was mutated to an *Nco*I site using the polymerase chain reaction (PCR) and the oligonucleotide 5′-AGCA*C*CATG*G*CACAGCAGCAGAGGATGACA-3′. This *Nco*I−*Spe*I (filled in) fragment was subcloned in to the *Nco*I and *Bst*EII (filled in) sites of pSP64-Xβm. Sequencing of the new construct confirmed that no mutations had been introduced by the PCR reaction. Capped trace-labelled message was produced as described in Hopwood *et al.* (1991).

RNA extraction and analysis

RNA was extracted from frozen oocytes, embryos or dissected pieces as described in Gurdon *et al.* (1985). Northern blots were made, hybridized, washed and stripped as described by Hopwood *et al.* (1989a). Probes were made by random priming the XE12 *Hin*dII fragment from 2184 to 4061. A single stranded DNA probe for detecting injected XE12 and XMyoD RNA was made as described in Hopwood *et al.* (1991).

RNase protection assays were performed as described in Krieg and Melton (1986). All analyses with the exception of the 5S RNA ones were performed on the total RNA equivalent to one oocyte or embryo. For the 5S RNA analyses, 1/200th of amount of total RNA was used. Following hybridization RNA digestion was performed with RNases T1 and A.

The probe used to detect both XE12 and XE47 was a *Hin*dII−*Bgl*II fragment of the XE47 clone from 1805 to 2184 subcloned in pBluescript SK$^-$. Templates were made using T3 RNA polymerase after linearizing the construct with *Hin*fI. The 5S RNA probe was as described in Mohun *et al.* (1984).

Protein extracts

Embryo extracts were made as previously described (Taylor *et al.*, 1991). Tail regions of tadpoles are particularly rich in XMyoD (Hopwood *et al.*, 1992) and where they have been used in experiments here, 40 tail pieces were extracted into a total of 70 μl of buffer.

In vitro translation

Synthetic RNAs (20 μg/ml) were translated *in vitro* in rabbit reticulocyte lysate (purchased from R.T.Hunt, Dept of Biochemistry, University of Cambridge) as described by Jackson and Hunt (1983). The translation products were resolved and quantified by SDS−PAGE (Laemmli, 1970)

Electrophoretic mobility shift assays

These were essentially as described in Taylor *et al.* (1991) with specific details as follows. Binding reactions were performed without magnesium and protein mixing was for 5 min at room temperature prior to the binding reaction. Antibodies were added for 15 min on ice to immunodeplete the binding complex. The probe was then added for a further 15 min and incubated at room temperature. The XMyoD antiserum was as in Taylor *et al.* (1991), while the XE12 antiserum was kindly given by Cornelis Murre (Murre *et al.*, 1989b).

Embryos and RNA injection.

Embryos were prepared, cultured, injected and dissected as described in Hopwood *et al.* (1991). Due to variations in batches of embryos, probably reflecting differences in the rate of RNA degradation, all comparisons were made between samples from the same experiment.

Immunohistochemistry

Embryos were processed for histology and antibodies were used as described in Hopwood and Gurdon (1990).

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