Different regulatory elements are required for cell-type and stage specific expression of the Xenopus laevis skeletal muscle actin gene upon injection in X.laevis oocytes and embryos

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ABSTRACT
In the present study, we demonstrate by transcript mapping that the injected Xenopus skeletal muscle α-actin gene is transcribed and spliced in Xenopus oocytes but not correctly initiated at the α-actin promoter. This leads to correctly spliced transcripts even if constructs without putative promoter sequences are injected. On the other hand, α-actin transcripts are translated in injected oocytes as shown by the detection of α-actin protein. By contrast, correctly initiated α-actin transcripts can be found in neurula embryos when the injected clone contains 5' flanking sequences extending from +27 to -680. α-actin gene fragments without the 680 nucleotides 5' flanking region are activated unspecifically after midblastula transition, whereas the clones carrying this region are activated correctly at the end of gastrulation. Cell type specific expression seems to be modulated by sequences within the transcribed region.

INTRODUCTION
The actins are highly conserved contractile proteins present in all eucaryotic cells. In vertebrates three main groups of actin isoforms, α-, β-, and γ-actins have been identified (1). The β- and γ-actins are ubiquitous proteins of the cytoskeleton, whereas the α-actin genes are only expressed in muscle cells where they constitute a major component of the contractile apparatus (2). It is well established that among vertebrates the different members of this gene family are activated in a developmentally regulated and/or tissue specific manner (3). During Xenopus early embryogenesis the expression of the cytoskeletal actin starts after midblastula transition concomitant with the onset of RNA polymerase II transcription. However, a Xenopus cytoskeletal actin gene was found recently to be coexpressed with sarcomeric actins exclusively in muscle cells of late neurula stages (4). Cardiac and skeletal α-actin genes of Xenopus are
transcriptionally activated at the end of gastrulation in the area of the mesoderm which differentiates embryonic muscle (5). Recently, it was demonstrated that cloned cardiac actin genes from X. laevis and X. borealis injected into fertilized eggs of X. laevis are expressed in a cell type specific way and that 5' flanking sequences are required for specific expression (6, 7). Similar results were obtained with Dictyostelium actin 6 (8) and the human cardiac actin gene (9) transfected into cultured cells. Injection in Xenopus oocytes has been used for a wide range of studies on the expression of cloned DNA templates. Pol II genes injected into oocytes are expressed with a wide range of efficiency and accuracy, both on the RNA and protein level (10). One of the most extensively studied gene families are the histone genes. Some characteristics of these genes (no introns) favour them for expression studies (11, 12). In this study, the expression of injected Xenopus laevis skeletal actin genes in Xenopus oocytes and embryos was examined. Subclones containing or lacking the 5' flanking region and as well intron as exon sequences were used to determine a potential function of these sequences in the regulation of transcription and processing.

MATERIAL AND METHODS
Injection into oocytes and fertilized eggs of Xenopus laevis
Oocytes were obtained from adult Xenopus laevis females and injected with 25-30 nl DNA solution (100 ng/µl) aiming for the oocyte nucleus (13). For protein labelling experiments individual oocytes were injected with 60 nl $^{35}$S-methionine (10 µCi/µl) into the cytoplasm (14).

Eggs of X. laevis were in vitro fertilized according to the procedure described by Rusconi and Schaffner (15). After removal of the jelly coat by treatment with 0.2% cysteine-HCl (16), fertilized eggs were injected with 200 pg DNA in a volume of 20 nl.

RNA preparation from oocytes and embryos
Following incubation for different periods of time oocytes or embryos were frozen at -70°C and RNA extracted by the proteinase K method (16). Embryos were homogenized in 60 µl buffer (1 mg proteinase K/ml, 10 mM Tris, pH 7.5, 1 mM MgCl$_2$, 100 mM NaCl, 2% (w/v) SDS)/embryo, and further processed as described for oocytes.
RNA preparation from skeletal muscle and kidney cells of *X. laevis*

Cultured *Xenopus* kidney cells (17) and skeletal muscle tissue of adult *X. laevis* were homogenized in a buffer containing 4 M guanidinium thiocyanate, 0.1 M Tris-HCl (pH 7.5), 2% sarcosyl, 7% mercaptoethanol (18). RNA was then purified from the lysate by centrifugation through a 5.7 M CsCl-cushion in a SW60-rotor for 16 hours at 30,000 min⁻¹ (19).

**Protein extraction and 2-dimensional gel electrophoresis**

For protein extraction, oocytes were homogenized in 50 μl buffer (9.5 M urea, 2% (w/v) Nonidet P-40 (Fluka, Neu-Ulm, FRG), 2% ampholines (1.6% pH 5 - 7, 0.4% pH 3 - 10; Serva, Heidelberg, FRG), 5% mercaptoethanol, 0.25% SDS) per oocyte. The supernatant was run on 2-dimensional gel electrophoresis according to O'Farrell (20). In the first dimension the equivalent of proteins extracted from one oocyte was loaded onto each gel. The gels were run at 400 V for 19 hours. For the second dimension we used 12% polyacrylamide gels and 3% stacking gels (21). The gels were stained with coomassie blue. For fluorography the gels were treated with NEN EN³HANCE for one hour, dried on Whatmann 3MM paper in a vacuum slab gel drier and exposed for 2 days at -70°C.

**Nuclease S1 protection assays**

RNA of 4 - 5 oocytes or embryos was hybridized for 12 hrs against 5,000 - 10,000 cpm DNA fragment which had been labelled at the 5' end with ³²P (0.5 - 1.5 x 10⁶ cpm/pMol) in 20 μl 80% FA, 0.4 M NaCl, 40 mM PIPES (pH 6.4), 1 mM EDTA (22). The hybridization temperature was 42°C for the 750 bp Hpa II fragment from α3HK600 and 50°C for the 480 bp Hpa II fragment from α3BP. For the internal RNA standard the RNA of 0.3 - 0.5 oocytes or embryos was hybridized at 57°C with the 293 bp Ava II fragment from a *X. laevis* rDNA subclone, pXL1168. For this clone a 1168 bp Alu I fragment of pXL101A (23) containing sequences of the nontranscribed spacer, the external transcribed Spacer and 18S rRNA was cloned into pBR322 (16). The hybridization was performed in a 10 μl volume and then added to the corresponding samples hybridized with the 750 bp Hpa II or 480 bp Hpa II fragment. The combined samples were digested with 60 - 80 units nuclease S1 (Sigma München) and analyzed on 7% polyacrylamide sequencing gels (16).
Dissection of Embryos

48 h old \( X.\) laevis embryos (stage 34) (24) were kept on ice for some minutes and dissected with a sharp blade into a part containing the dorsal muscles and a "non muscle part".

RESULTS

The \( X.\) laevis skeletal \( \alpha\)-actin clone \( a3\) and derived subclones

The skeletal muscle \( \alpha\)-actin gene used in this experiments was isolated from a \( \lambda\) phage library and subcloned into pUC8 as described (24) (Fig. 1a). The first intron which is 1.5 kb in length interrupts the leader region 41 bp downstream of the transcription initiation site (Fig. 1b). For studies of control sequences several fragments of the 5' region were subcloned. A 2.6 kb Bam HI-Hind III fragment containing 660 bp 5' flanking sequences, exon I and II, intron I and a part from intron II was subcloned into pUC8 (a3BH) (Fig. 1c). For the subclone a3BP a 700 bp Bam HI-Pst I fragment of a3BH which consists of 680 bp 5' flanking sequences and 27 bp of the first exon was cloned into pUC8 (Fig. 1e) and for a3PH a 1.9 kb Pst I-Hind III fragment lacking the start point of transcription was subcloned (Fig. 1d). For characterization of \( \alpha\)-actin transcripts a 600 bp Kpn I-Hind III restriction fragment of a3 carrying exon II and flanking intron sequences was subcloned into pUC8 (Fig. 1f). This fragment was cloned in opposite orientation compared to the other clones described above. Digestion of this plasmid with Hpa II generates a 750 bp fragment consisting of 350 bp vector sequences, 300 bp intron I and the first 91 bp of exon II (Fig. 2B a). This Hpa II fragment was labelled at the 5' end and used as a probe in nuclease S1 protection experiments (Fig. 2B b). Annealing of this end labelled probe to various transcription products of the \( \alpha\)-actin gene and subsequent digestion by nuclease S1 is expected to generate two S1 resistant fragments: one 400 bp fragment protected by the \( \alpha\)-actin primary transcript and a 91 bp fragment protected by the spliced \( \alpha\)-actin mRNA (Fig. 2B c and d).

Transcription of the \( a3\) actin gene in injected oocytes

After injection of \( a3\), which carries the whole skeletal muscle gene (24), into Xenopus oocytes, the RNA was analyzed by nuclease S1 protection using the 750bp Hpa II fragment from a3HK600 as
Fig. 1. The *X. laevis* skeletal muscle α-actin clones.
For the genomic clone α3 a 9.8 kb Eco RI fragment containing the complete α-actin gene was isolated from a genomic λ phage library of *X. laevis* and cloned into pUC8 (25) (a). A 3.8 kb Eco RI-Hind III fragment contains a part of the 5' flanking region, exon I and II, intron I and a part of intron II (b). α3BH contains the 2.6 kb Bam HI-Hind III fragment (c), α3PH the 1.9 kb Pst I-Hind III fragment (d) and α3BP the 0.7 kb Bam HI-Pst I fragment (e) inserted into the multiple cloning site of pUC8. The 0.6 kb-Kpn I-Hind III fragment containing exon I and adjacent intron sequences was cloned with Bam HI linkers into the Bam HI site of pUC8 generating α3HK600 (f). (b-f) white boxes: 5' flanking region, hatched boxes: 5' untranslated region, dotted boxes: intron sequences, black boxes: coding region. E: Eco RI, B: Bam HI, H: Hind III, P: Pst I, Hp: Hpa II, K: Kpn I. Only the Hpa II sites relevant for the cloning procedure are shown.

probe. We used a probe which maps at the second exon because hybrids to the 41 bp short exon I turned out to be unstable for standard S1 analysis. A further advantage the 750 bp fragment is the possibility to discriminate between primary transcript and spliced mRNA. During incubation (3, 8 and 20 h) an increasing amount of a 91 nucleotide fragment is detectable which is expected to be protected by spliced α-actin mRNA. Precursor α-actin RNA is identified as a 400 nucleotide fragment (Fig. 2A 4 – 6).
Fig. 2. A) Nuclease S1 analysis of transcripts after injection of X. laevis α-actin clone a3. The 5' labelled 750 bp Hpa II fragment from a3HK600 was hybridized against RNA from four non-injected (1) or injected oocytes (2 - 7), 16 μg tRNA (8), 16 μg kidney cell RNA (9) or 20 μg skeletal muscle cell RNA (10). The oocytes were injected with 6 ng pBR322 (2) or 3 ng a3 (3 - 7). In one case 5 ng α-amanitin was coinjected (7). The oocytes were immediately frozen after injection (3) or incubated at 18°C for 3 (4), 8 (5) and 20 hours (6, 7). The further procedure is described in materials and methods. fl: 750 bp full length probe, pt: 400 bp fragment protected by primary transcript, sp: 91 bp fragment protected by spliced mRNA. Length marker: pBR322/Hpa II (M1) and pBR322/Hpa II + pBR322/Alu I (M2). B) S1 hybridization probe and protected fragments. The 600 bp Hind II-Kpn I fragment of a3 was subcloned into the Bam HI site of pUC8 (a).

Despite the high homology of the coding regions of the different Xenopus actin genes we could not observe any protected fragments in non-injected oocytes or oocytes immediately frozen after in-
Fig. 3. Transcripts of injected a3 are translated.
A - C) Two-dimensional gel electrophoresis of proteins.
A) Proteins of one oocyte injected with 60 nl 35S-methionine into the cytoplasm and incubated for 24 hours, were separated by two-dimensional gel electrophoresis and stained with coomassie blue.
B) Autoradiogram of the same gel.
C) Proteins of one oocyte, which had been injected with 3 ng a3 into the nucleus and 60 nl 35S-methionine into the cytoplasm, were separated after incubation for 24 hours by two dimensional gel electrophoresis and visualized by autoradiography.
D) RNA from five oocytes of the same batches, which were used for protein extraction, were analysed by nuclease S1 mapping. Oocytes were noninjected (1), injected with a3 (2), with 35S-methionine (3 and 5) or with both (4 and 6) and incubated for either 24 hours (1 - 4) or 48 hours (5 and 6).

injection, which also shows that there is no hybridisation of the probe with the injected DNA (Fig. 2A 1 and 3). After injection of pBR322 we consistently observed bands with the length of about 150 nucleotides caused by the 5' labelled non-coding strand of the 750 bp hybridization probe which has a 150 nucleotide homology to pBR322 transcripts (Fig. 2A 2). After coinjection of α-amanitin the 91 and the 400 nucleotide band disappears (Fig. 2A 7). This shows clearly that the injected gene is transcribed by an α-amanitin sensitive RNA polymerase. As a positive control we hybridized the fragment against total RNA from Xenopus laevis muscle cells (Fig. 2A 10). The protected fragment migrates to the position expected for the spliced mRNA but no signal for the unspliced transcripts is found. For a clear demonstration that there is no cross-hybridization with cytoskeletal actin mRNA we analyzed total RNA of Xenopus kidney cells (Fig. 2A 9).
**α3-actin transcripts are correctly translated**

To determine whether transcripts of α3 were translated, proteins from injected oocytes were analyzed on 2-dimensional gels. Figure 3A shows a coomassie blue stained gel of control oocytes which had been injected with 35S-methionine. A prominent β- and/or γ-actin spot can be detected. A similar spot is identified on the corresponding autoradiograph (Fig. 3B). Oocytes injected with α3 DNA and 35S-methionine produced two additional spots, one corresponding to the α-actin (Fig. 3C). In a parallel experiment, we extracted the RNA from oocytes of the same batches and analyzed the transcripts by the nuclease S1 protection assay (Fig. 3D). Spliced α-actin transcripts can be detected after 24 and 48 hours (lanes 4 and 6) in double injected oocytes and after 24 hours in oocytes injected with α3 (lane 2), but not in control oocytes (lanes 1, 3 and 5).

**Transcription of the α3 subclones**

To elucidate the role of upstream sequences in the regulation of the Xenopus α-actin gene, we injected different α3 subclones.
Clone α3BH contains the transcription start site and 5' flanking sequences (Fig. 1). Subclone α3PH does not contain the region from -680 to +27 (Fig. 1). After injection, the oocytes were cultured for 24 hours and the RNA was analyzed. Whether there is the α-actin initiation site on the injected plasmids (α3, α3BH; Fig. 4B and 4C) or not (α3PH; Fig. 4D) in all cases transcripts carrying the intron I sequences (pt) or transcripts which were spliced at the intron I/exon II boundary (sp) could be detected. In the case of α3HK600 carrying only exon II and some adjacent intron sequences, the splice product (sp) is observed together with the full length protected probe (Fig. 4E). This indicates that the promoter sequences of the α-actin gene are not necessary for transcription of oocyte injected α-actin DNA. (c.f. also Fig. 6).

In the case of the subclones α3BH and α3PH an additional strong band with 314 nucleotide length appears (Fig. 4C and 4D). Because the 600 bp Kpn I - Hind III fragment is inserted into pUC8 in the opposite orientation compared to the other actin clones the 5' labelled non-coding strand of the 750 bp Hpa II fragment hybridizes against readthrough transcripts of the α-actin gene and is protected up to the used cloning site of the plasmid. The corresponding signal is observed in some batches of oocytes after injection of the full length clone α3, presumably because of readthrough beyond the Pol II termination signal but it is by far stronger after injection of α3BH and α3PH which lack the 3'end of the actin gene. We have no precise explanation for the additional strong 335 nucleotide signal, which was reproducibly observed after injection of α3PH. In oocytes injected with pUC8, a signal in this region is observed deriving from the homology between pUC8 and the probe (data not shown). Whether the signal in the case of α3PH is derived by using a cryptic acceptor site in the intron I or by splicing out the whole actin gene sequences remains to be analyzed in detail. To make sure that the amounts of RNA extracted from the oocytes are comparable, we hybridized a 293 bp Ava II fragment from pXl1168 against the 40S precursor rRNA of the oocytes (16). The endogenous rRNA protects a 44 nucleotide fragment which can be used as an internal standard for the extracted RNA.
Fig. 5. A) Transcription of the α-actin subclones after injection into fertilized eggs.
In vitro fertilized eggs were not injected (1) or injected with the 9.8 kb Eco RI fragment of α3 (2), α3 (3), α3BH before the first cell division (4), in one blastomere of the two cell stage (4') and α3PH (5). The embryo were allowed to develop at 21°C and frozen at the indicated developmental stage. The amount of injected DNA was 200 pg and RNA of five embryos was analyzed with the 750 bp Hpa II fragment. st 7: early blastula, st 9: late blastula, st 11: gastrula, st 20: neurula.
B) Transcript analysis from dissected embryos.
6 embryos, 48 hours old (stage 34) (24) injected with α3BH (2, 3) and α3PH (4, 5) were dissected into a "muscle" fraction (2, 5) and a "non muscle" fraction (3, 4). RNA from 6 whole uninjected embryos was analyzed in lane 1. Length marker: pBR322/Hpa II (M).

Transcription of the α-actin clones after injection into fertilized eggs

In addition to the oocyte injection experiments we studied the transcriptional behaviour of the α3 subclones after injection into fertilized eggs. In a number of investigations it has been shown that most genes injected into fertilized eggs are initiated
at the correct start site but in an unspecific manner after mid-blastula transition (see discussion). With the 750 bp probe no transcription was detected before neurula stage (stage 20) in uninjected embryos (Fig. 5A 1), or when linear Eco RI insert of a3 (Fig. 5A 2), a3 (Fig. 5A 3) and a3BH (Fig. 5A 4 and 4') was injected, whereas transcripts from the promoterless actin subclone a3PH (Fig. 5A 5) can be detected in stage 11 embryos. In a3 injected embryos it is not possible to discriminate between endogenous a-actin transcripts and transcripts of the injected DNA. An indication that the injected a-actin template is transcribed in neurula embryos is the higher amount of spliced product (91 nucleotide band) in a3 injected embryos and the presence of the primary transcript (400 nucleotide band) which is absent in the uninjected control. A clear proof for the transcription of the injected a-actin DNA is the presence of the 314 nucleotide band in a3BH injected embryos and the 314 and 335 nucleotide band in a3PH injected embryos. These results could be confirmed with eggs from different females. These data suggest that 5' flanking regions extending from the start point of transcription to -680 are necessary for the stage specific regulation of the a-actin gene. Injected a-actin genes have been shown to be exclusively expressed in muscle tissue (6, 7). To investigate if the expression of the injected skeletal muscle actin gene is also restricted to muscle cells, we dissected 48 hours old X. laevis embryos (stage 34) into "muscle" and "non muscle" fraction and analyzed the RNA with the 750 bp Hpa II fragment. a3BH and a3PH are exclusively expressed in muscle tissue (Fig. 5B 2 - 5) as can be seen by the presence of the 314 nucleotide band. The 335 nucleotide band was absent when a3PH was injected. The restriction of a3PH transcripts to muscle cells is remarkable because cap site and 5' flanking sequences are missing in this construct, indicating that the tissue specificity and stage specific activation are governed by different control sequences.

Initiation of a-actin transcription in oocytes and embryos
To determine whether the initiation of the adjacent a-actin gene is correct, we injected the a3BP subclone containing 5' flanking sequences and 27 nucleotides of the exon I (Fig. 1e). With a 480 nucleotide Hpa II probe it is possible to detect correctly ini-
Fig. 6. Initiation of the injected α-actin gene.
A) The hybridization probe used for nuclease S1 assay was a 480 bp Hpa II fragment (b) isolated from α3BP (a). Correctly initiated transcripts protect a 350 nucleotide fragment (c). A 324 nucleotide fragment is protected by pUC8 transcripts (d).

S1 analysis after injection of α3BP into oocytes (B) and embryos (C).

B) Oocytes were not injected (1) injected with pUC8 (100 ng/μl) (2) or α3BP (100 ng/μl) (3) and incubated for 24 hours at 18°C. RNA equivalents of five oocytes were hybridized with the 480 bp Hpa II fragment of α3 bp and RNA of 0.5 oocytes with the 293 bp Ava II fragment. For negative controls RNA of five 48 h old embryos (4) was used for hybridization. In lane 5 the reactions were performed in the absence of RNA. (6): the full length hybridization probe.

C) RNA from 6 embryos, non-injected (1, 3 and 5) or injected with 200 pg α3PB (2, 4 and 6) was extracted 8, 12 and 24 hours after fertilization. An RNA equivalent of 5 embryos was used for the hybridization with the 480 bp Hpa II fragment and of 0.5 embryos for the internal RNA standard (293 bp Ava II fragment). Length marker: pBR322/Hpa II (M).

Initiated transcripts of the injected template without cross reaction of endogenous α-actin mRNA.

In oocytes, no correct α-actin transcripts can be detected (Fig. 6B 3). Similar results were obtained after injection of α3 with
other S1 probes and primer extension experiments (data not shown). Transcripts from injected pUC8 initiated further upstream of the multiple cloning site and protect a 324 nucleotide fragment which corresponds to the plasmid portion of the hybridization probe (Fig. 6A and 6B 2). No protected fragments can be observed in non-injected oocytes (Fig. 6B 1). This indicates that there is no crossreaction of the hybridization probe with oocyte RNA.

To make sure that the 480 nucleotide fragment does not hybridize to endogenous α-actin mRNA we hybridized it against total RNA of 48 hours old Xenopus embryos (Fig. 6B 4) in which the α-actin genes are expressed (Fig. 5). For similar results see also (5).

To exclude artificial bands caused by the probe we performed hybridization and S1 digestion in the absence of RNA (Fig. 6B 5).

In contrast to the incorrect initiation in oocytes correctly started transcripts of the injected α3BP can be detected in neurula embryos. These transcripts protect a 350 nucleotide-fragment of the S1 probe (Fig. 6C).

DISCUSSION

In the present study we present evidence that the cloned X. laevis skeletal muscle α-actin gene is transcribed although transcripts are not correctly indicated, after injection into oocytes. For an avian keratin and a X. laevis cardiac α-actin gene incorrect transcripts as well as correctly initiated mRNAs have been described (6, 26). No correctly initiated transcripts of the injected skeletal muscle α-actin DNA are detectable in oocytes. The fact that they are present in injected embryos indicates that there are factors available in the embryo mediating correct initiation which are absent or inactive in oocytes. It is well known that in oocytes promotor-like sequences located in pBR322 can function as initiation sites of RNA polymerase II transcription (27). This is an explanation for the expression of injected RNA polymerase II genes without correct initiation of transcription. Most of these unspecific initiation events seem to be suppressed in the developing embryo (for discussion see 10). A further result of our study is the observation that the first intron of the skeletal α-actin gene is excised using the correct acceptor site.
This is in agreement with a wide range of studies where correct splicing was observed in oocyte injection experiments, e.g. in coupled transcription-translation assays and in RNA injection experiments (for review see 14). However, correct splicing is not always observed. Expression of the ribosomal protein L1 gene was found to be regulated by incomplete splicing (28) and no splicing of transcripts of an avian keratin gene was observed (26).

A clear proof for the correct processing of the primary transcript to a functional mRNA is the appearance of a protein migrating at the same position as a-actin after injection of the cloned a-actin DNA. The amount of the synthesized a-actin protein deriving from the injected DNA is comparable with the high levels of newly synthesized endogenous β- and γ-actin proteins. From this we can conclude that the expression of a-actin is not regulated at the level of translation. A similar conclusion had been obtained by Sturgess and coworkers, from injection of tailbud poly-A⁺-RNA into oocytes (29).

From embryo injection experiments it had been shown that a number of templates such as SV40 (30), HSV thymidine kinase (our unpublished observation), Drosophila alcohol dehydrogenase (30), rabbit β-globin (15) and Xenopus adult β-globin genes (31) start to be transcribed at midblastula transition where a general onset of transcription occurs (32 - 34). In this unspecific way the a3 subclone without the region +27 - -680 (a3PH) is activated whereas the promoter containing clone a3BH behaves like the endogenous gene. This result is in agreement with investigations of other developmentally regulated embryonic genes like the gastrula specific gene GS17 and the tadpole β-globin gene (31, 35). Interestingly, both clones a3BH and a3PH are expressed exclusively in muscle tissue of 48 hours old X. laevis embryos (stage 34) indicating that the cell type specific expression of the gene is modulated by sequences other than the 5' flanking region. It has been shown that sequences within the transcribed region can play an essential role in the regulation of Pol II genes (26, 36). More detailed studies have to be focused on the precise identification of these regulatory sequences.
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