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Identification and developmental expression of the *Xenopus laevis* cystic fibrosis transmembrane conductance regulator gene

Stephen J.Tucker, David Tannahill[1] and Christopher F.Higgins*

Imperial Cancer Research Fund Laboratories, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU and [1]ICRF Developmental Biology Unit, Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK

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ABSTRACT

An amphibian homologue of the human cystic fibrosis transmembrane conductance regulator (CFTR) gene has been isolated from *Xenopus laevis* by polymerase chain reaction (PCR) amplification. The 4455bp sequence encodes a predicted polypeptide of 1485 amino acids which has an overall homology at the amino acid level of 77% identity and 88% similarity with human CFTR. Comparison of these evolutionarily diverse CFTR sequences has structure-function implications. Investigation of the expression of the *Xenopus* gene during early stages of development (Stages 1–48), using RNAase protection assays and PCR analysis of total *Xenopus* RNA, shows CFTR mRNA to be present at the very earliest stages of development, including the oocyte and blastula stages, with increasing amounts during subsequent development. The identification of mRNA for a CFTR homologue in the *Xenopus* oocyte and early stages of development has implications for its biological role.

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in the Caucasian population. The gene responsible has been identified and shown to encode a polypeptide of 1480 amino acids, designated the cystic fibrosis transmembrane conductance regulator, CFTR (1)(2). CFTR is organised as two related halves, each consisting of a transmembrane domain with six putative membrane-spanning segments and a nucleotide-binding fold. The two halves of the molecule are separated by a regulatory R-domain which is highly charged and contains consensus phosphorylation sites for cAMP-dependent protein kinase A (3). The CFTR polypeptide shows structural and sequence similarities to the ATP-binding cassette (ABC) superfamily of proteins which includes the multi-drug resistance (MDR) P-glycoproteins and many other proteins associated with active transport processes (4). The CF gene is specifically expressed in secretory epithelial cells, generally implicated in the pathophysiology of the disease, including epithelia of the pancreas, colon, sweat ducts and genital ducts (5)(6).

Defects in anion permeability of secretory epithelia have been implicated in CF pathophysiology (7) and the CF gene has been shown to correct chloride channel defects when expressed in cell lines derived from CF patients (8)(9). Recent studies have shown that overexpression of human CFTR in *Xenopus* oocytes and in mammalian and insect cells, correlates with the appearance of a cAMP-dependant, small conductance, chloride channel (10)(11)(12). This, combined with the demonstration that mutations in CFTR can alter the ion-selectivity of the channel (13), and the functional reconstitution of CFTR into lipid bilayers (14), provides strong evidence that the CFTR gene product possesses intrinsic chloride channel activity. Whether or not this channel activity is the only function of CFTR remains to be ascertained.

As *Xenopus laevis* is a well established model system for studying development, as well as an expression system of choice for the electrophysiological characterisation of channel proteins such as CFTR (12)(15), the possibility that this organism possesses a CFTR homologue is of particular relevance. In this paper we describe the identification and characterisation of a *Xenopus* CFTR homologue and analysis of its expression during early development.

RESULTS

Initial identification of a putative *Xenopus* CFTR homologue

Comparison of the only two CFTR sequences available at the initiation of this study, for human and mouse (1)(16), allowed short regions of conserved amino acid sequence flanking the R-domain (CVCKLMAN and EINEEDLK) to be identified. Degenerate oligonucleotide primers were designed to amplify the intervening region using the polymerase chain reaction (PCR). This region was chosen because the R-domain is unique to CFTR amongst the ABC-transporter proteins, and because the R-domain is encoded as a single exon in human and mouse. A fragment of the predicted size (700bp) was amplified from *Xenopus* genomic DNA using these primers. This amplified fragment was cloned and nucleotide sequence analysis showed the deduced amino acid sequence to share 65% identity to the R-domain of human CFTR.

To confirm this amplified fragment was indeed *Xenopus* DNA, a Southern blot of *Xenopus* genomic DNA, digested with several

* To whom correspondence should be addressed

different restriction enzymes was probed with this R-domain fragment and washed at high stringency. The characteristic band patterns of single-copy genes from tetraploid organisms such as *Xenopus* were observed (data not shown).

Isolation of cDNAs encoding the entire *Xenopus* CFTR

The remainder of the *Xenopus* CFTR gene was amplified from cDNA made by reverse transcription of late stage (stage 44/48) total RNA. Using one specific PCR primer (based on the nucleotide sequence of the *Xenopus* R-domain, above) and one degenerate primer (based on conserved amino acids in human and mouse CFTR), overlapping fragments of the predicted size were amplified by PCR and cloned. Sequencing showed them to be closely related to the regions flanking the R-domain in human CFTR. Using this sequential approach a total of 3 fragments upstream and 4 downstream, of the initial R-domain clone were amplified, cloned and sequenced (see Figure 1 for details). These 8 clones contained the whole coding sequence for the *Xenopus* CFTR homologue.

Nucleotide sequence and deduced amino acid structure of *Xenopus* CFTR

Alignment of the nucleotide sequences of the ovelapping clones gave a continuous sequence of 4455bp. The deduced amino acid sequence predicts a 1485 amino acid polypeptide showing an overall sequence identity of 77.4% to human CFTR with an overall similarity of 88.3%. The nucleotide sequences share 73% identity. Table 1 presents the sequence identity between human and *Xenopus* proteins on a domain basis. Alignment of the amino acid sequence with the sequences of the four other published CFTR homologues (1)(16)(17)(18) (Figure 2) reveals regions of high sequence conservation and regions of high variability.

Developmental expression of *Xenopus* CFTR

PCR analysis. Total RNA samples were obtained from 11 different developmental stages of *Xenopus* embryos (19). cDNA copies of these RNA samples were prepared by reverse transcription with random oligonucleotide priming. High stringency PCR, using primers to the uniformly expressed ornithine decarboxylase (ODC) gene, was used to quantitate the amount of cDNA in each sample. Using the same sample, equal amounts of cDNA from each developmental stage were analysed by PCR using primers specific to the *Xenopus* CFTR gene. Figure 3 shows that mRNA for the *Xenopus* CFTR is detectable in the single cell egg stage, and throughout early development. The amount of message increases during later developmental stages, most rapidly around stage 32. A larger fragment of over 2Kb was also amplified in RNA from later stages of development and also hybridized with the *Xenopus* probe in a Southern blot (Figure 3). Due to the high stringency and sensitivity of this procedure it is likely that this represents unspliced mRNA and that the PCR primers span an intron-exon boundary.

RNAase protection analysis. As PCR-based methods are not entirely quantitative an antisense RNA probe against the R-domain was used in RNAase protection assays on total RNA samples from developmental stages 1, 6, 9, 11, 14, 18, 23, 32, 40, 44 & 48. A tRNA internal control to show probe specificity was also included and quantitation achieved with a probe to the uniformly expressed ODC gene. These data (Figure 4) confirm the conclusions obtained by PCR analysis, and show that *Xenopus* CFTR mRNA is present in the single-cell egg (stage-1) and increases most significantly from stage 32.

DISCUSSION

Structure-function relationships

The *Xenopus* homologue of the human CFTR gene has been isolated and sequenced. Southern blotting confirms that the cDNA isolated and characterised here is indeed derived from *Xenopus* and that it is a single copy gene. The deduced amino acid sequence of *Xenopus* CFTR is 77.4% identical and 88.3% similar to the human CFTR sequence. This compares with the mouse sequence (16) which shows 78.4% identity, the bovine sequence (17) 90% identity, and the dogfish sequence (18) 72% identity,

TABLE 1: Percentage identity and similarity of the individual domains of the *Xenopus* CFTR with the equivalent domains of human CFTR

	% Identity	% Similarity
First transmembrane domain	86.7	93.7
First NBF	87.0	94.2
R-domain	64.7	79.4
Second transmembrane domain	74.6	85.6
Second NBF	81.7	93.5
TOTAL	77.4	88.3

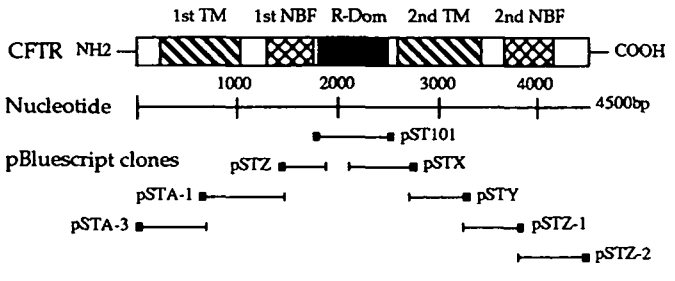

Figure 1. Cloning strategy for isolating the *Xenopus* CFTR homologue. The relative position of the pBluescript clones isolated in this study, compared with the domain structure of the protein (1), is shown. At the ends of each clone the type of primer used to amplify each fragment is indicated; a square representing a degenerate primer and vertical bar representing a specfic primer. The sequences of these primers are shown in Table 2.

TABLE 2: Primers used in the PCR amplification of the *Xenopus* CFTR gene to generate the clones indicated in Figure 1 . The sequences of the degenerate primers are given in the IUPAC nomenclature.

CLONE	5' PRIMER (5'-3')	3' PRIMER (5'-3')
pSTA-3	ATGCARMRIWSICCIYTNGA	CCAAACAAAATGAGCCAGAGC
pSTA-1	AAYYTIAAYAARTTYGAYGARGG	GTACTGGTCATAGGAAACGC
pSTZ	TGGATYATGCCHGGHACHATHAA	GCTTCCTTCGTGCAAAATAAG
pST101	TGYGTITGYAARYTIATGGCNAA	YTIARRTCYTCYTCRTTDATYTC
pSTX	TCCTCGCAAATCAAAGTC	ACDCCVACRTADATRTARAA
pSTY	GTATCTGAAGTATCAGACACC	TCDATNCKCATYTGRAACCA
pSTZ-1	CATACTGCAAACTGGTTTCTC	ATIACICCRAADGCYTTNCKCC
pSTZ-2	CAGACCATTCCACTTCAGAAATG	IARICKDGTITCYTGNACYTC

with human CFTR. A partial sequence of two membrane spanning regions of *Xenopus* CFTR reported previously (20) differs considerably from the sequence reported here. Such differences are unlikely to be the result of allelic variation or cloning induced artefacts. Comparison of the individual domains of CFTR (Table 1) shows that the first nucleotide-binding fold (NBF) is the most highly conserved between *Xenopus* and human. This is consistent with the observation that most mutations associated with CF in the human gene alter this domain, including the major mutation associated with the disease, ΔF508 (21). The first transmembrane domain is equally highly conserved. The R-domain is the least conserved of the individual domains sharing only 64.7% identity with the human sequence.

Comparison of *Xenopus* CFTR with the other four published CFTR homologues in figure 2 is instructive showing the *Xenopus* sequence to be most similar to that of the dogfish; both have an extra amino acid at position 61 when compared to the mammalian sequences, the extracellular loop between TM 7 and TM 8 is

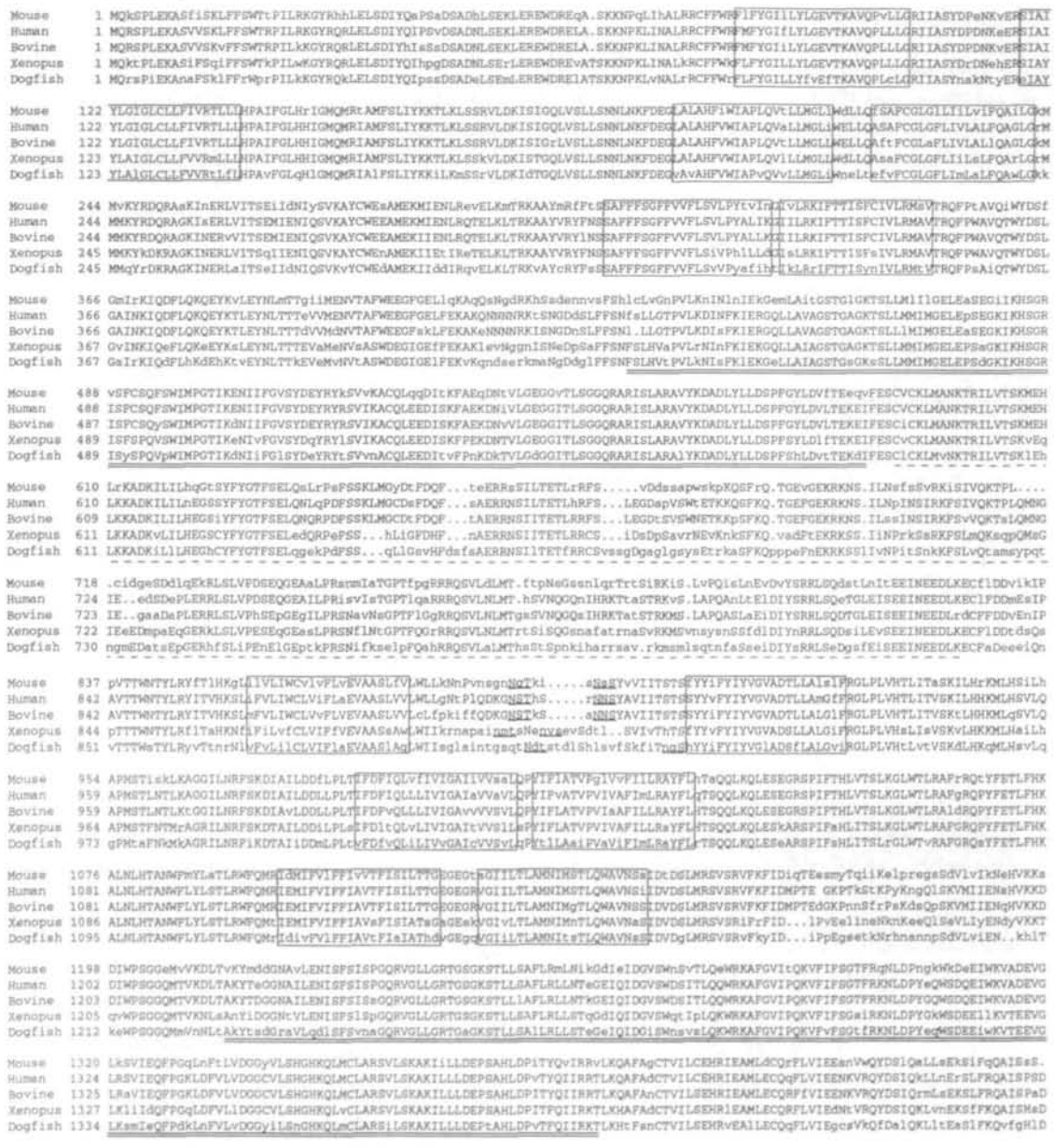

Figure 2. Alignment of the five published full-length sequences for CFTR (1)(17)(18)(16). Identical adjacent residues are in capital letters, those not are in the lower case. The first and last 8 amino acids in the *Xenopus* sequence correspond to the primers used and are in the lower case as well. The twelve regions predicted to span the membrane (TM 1−TM 12) (1) are boxed, the two nucleotide binding folds are double underlined, and the R-domain highlighted with a dashed line. The putative glycosylation sites between TM 7 and 8 are underlined in each of the five sequences.

longer in both proteins, and both have an equivalent deletion of two residues in the region after TM12 compared with the mammalian sequences (Δ1169−1170). All residue numbers refer to the human sequence (1) unless otherwise stated. In the first transmembrane domain, the predicted transmembrane segments (TM 1−6) are highly conserved in all five species, including lysines 95 and 335 which play a role in determining anion selectivity (13) (although the dogfish has the conservative substitution of an arginine in the equivalent to position 335. The extracellular loops linking the transmembrane segments are poorly conserved. In contrast, the cytoplasmic loops linking TM 2 to TM3 and TM 4 to TM 5 are highly conserved, pointing to an important functional role; in human P-glycoprotein and its homologues the equivalent cytoplasmic loops are equally highly conserved and a mutation (G185V) in the loop between TM2 and TM3 alters substrate specificity (22)(23).

The first nucleotide-binding fold includes the longest stretch of sequence identity between the five species (residues 540−580). Many missense mutations resulting in CF are clustered in this region (24). The region surrounding the site of the major mutation, ΔF508 (residues 495 to 515) is also highly conserved with only a few conservative substitutions between species. Other missense mutations implicated in the disease, A455E and G458V (25)(26), also show conservation at these positions, supporting the view that they are functionally important.

The R-domain is the most divergent amongst the five sequences, and this low degree of conservation is also seen in R-domain sequences from the monkey, sheep, guinea pig and rabbit (17). Despite the low degree of sequence conservation, protein kinase A (PKA) phosphorylation sites (consensus R-R/K-X-S) appear highly conserved. Three of the four PKA sites implicated in the regulation of the human CFTR chloride channel *in vivo*, serines 660, 795 and 813 (3) are conserved in the five sequences aligned in figure 2, with the fourth site (serine 737) having the sequence RHFS in the dogfish (18). Many other potential phoshorylation sites in this domain (1) exhibit varying degrees of conservation between species suggesting that the exact sites of phosphorylation may not be as critical as the overall charge of the domain which may be the regulating factor. Two potential PKA sites in the carboxyl tail of the dogfish protein (18) which are not present in the mammalian proteins, are conserved in *Xenopus*. They may represent additional PKA regulatory sites (18) or may simply reflect the conserved highly charged nature of this carboxyl tail to which phoshorylation would contribute.

In the second transmembrane domain the cytoplasmic loops linking TM 8 to TM 9 and TM 10 to TM 11 are highly conserved implying that, as for the first transmembrane domain, the cytoplasmic loops play an important functional role. In the extracellular loop between TM7 and TM8, sites for *N*-linked glycosylation have been identified in the human sequence (1). These consensus glycosylation sites (N-X-S/T) are conserved in all five species although the sequences in which they are embedded are highly variable. The region linking the second transmembrane domain to the second nucleotide-binding fold is also highly variable, both in length and sequence, suggesting its role may simply be as a 'flexible linker' with little other functional importance. This is in contrast to the equivalent region linking TM 6 to the first nucleotide-binding fold. The second nucleotide-binding fold of CFTR differs somewhat from the first, and to nucleotide-binding folds of other ABC transporters, and it has been suggested the two NBFs of CFTR might have different functions (10). Nevertheless, the conservation of this domain between the five species is high, especially around the Walker nucleotide binding motifs (27)(28) implying an equally important role as that of the first NBF.

Developmental expression of *Xenopus* CFTR

Two independent approaches, one PCR-based the other involving RNAase protection, show that CFTR mRNA is present in significant amounts at the very earliest stages of development. Since there is no new transcription until the mid-blastula stage (4000 cells), the finding of mRNA in the single-cell egg implies mRNA is also present in the oocyte. The amount of mRNA per cell does not increase significantly during development until around stage 32 correlating with the appearance of defined epithelial tissues in the larva. We do not know whether the CFTR mRNA in the oocyte is translated to protein since it is thought that some maternal mRNA is translationally inactive in the oocyte. Nevertheless, it is unlikely that after the mid-blastula stage (stage seven) when general transcription is initiated, that the CFTR protein is not made. The possible function of CFTR in these early stages of development is intriguing. In mammalian systems CFTR expression appears to be restricted to secretory epithelial cells (5)(6) yet equivalent tissues do not appear in *Xenopus* until much later in development (stage 32). Analysis of human CFTR

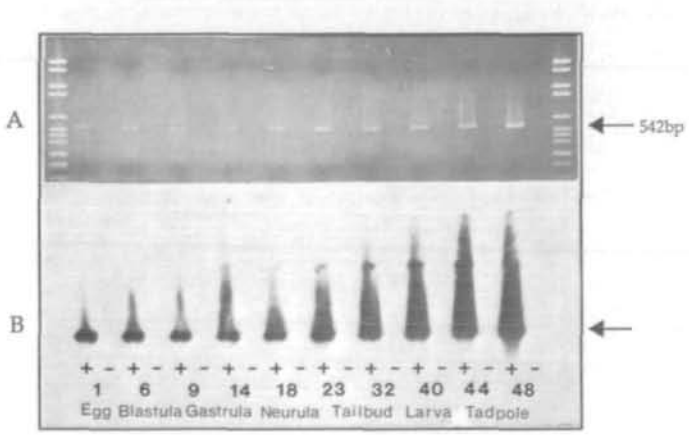

Figure 3. mRNA analysis by RT-PCR. Developmental stages are indicated by their stage numbers and equivalent morphology (19). (+) indicates with reverse transcriptase, (−) indicates without. Panel A: RT-PCR products after gel electrophoresis. The 542bp product is clearly seen in stage 1 and all subsequent stages. B: a Southern blot of the same gel probed with the corresponding *Xenopus* R-domain insert from clone pSTX.

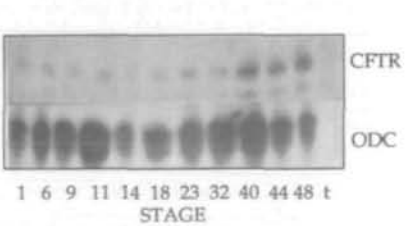

Figure 4. mRNA analysis by RNAase protection analysis. mRNA for the gene is clearly detectable in the egg stage 1 of development with the amount of mRNA increasing most significantly after stage 32. The ODC gene is indicated underneath to provide an internal control for quantitating the amount of mRNA tested. The tRNA internal control is shown in track 't'.

expression in mid-trimester fetal tissues has shown the gene to be expressed in ductal epithelia before they are fully differentiated (29). However, in *Xenopus*, at these much earlier stages, although cells have adopted a particular fate, they are relatively undifferentiated in morphological terms. If CFTR functions as a chloride channel in these cells, regulating anion balance, then this general function must become restricted to specific cell types during development. Electrophysiological studies on *Xenopus* oocytes have not identified an endogenous chloride channel with characteristics similar to those observed for human CFTR (12) (15). Alternatively, the *Xenopus* CFTR may have a different function in these early developmental stages. The finding that the related P-glycoprotein has independent transport and chloride channel functions (30) raises the possibility that an alternative fuction may also be associated with CFTR.

MATERIALS AND METHODS

DNA manipulations and electrophoresis

DNA manipulations, agarose gel electrophoresis, Southern transfer and hybridisation were carried out by standard procedures (31). DNA ligase, Klenow fragment of DNA polymerase, and restriction endonucleases were from Boehringer and used according to the manufacturers instructions.

PCR amplification from genomic DNA

The R-domain of *Xenopus* was amplified from *Xenopus* genomic DNA using 40 PCR cycles (each of 95°C for 1min, 55°C for 1.5 mins and 72°C for 5mins) with 250ng template DNA, 1μg of each of the degenerate oligonucleotide primers X1 (TGYGTITGYAARYTIATGGCNAA) and X5 (YTTIARRTCYTCYT-CRTTDAYTC), 3U of AmpliTaq polymerase (Cetus), and 0.25mM dNTP's in 50μl total reaction buffer (10× Cetus reaction buffer is: 100mM Tris-HCl pH 8.3, 500mM KCl, 25mM $MgCl_2$, 0.01%(w/v) gelatin). PCR products were resolved on 0.8% agarose gels and the appropriate sized 700bp fragment excised and blunt-end ligated into pBluescript SK^+ (Stratagene) digested with *Eco*RV.

Southern blot analysis

15μg of *Xenopus* genomic DNA was digested, separately, with 100Units of various restriction endonucleases at 37°C. Digested DNA was resolved on 0.8% agarose gel and transferred a Hybond-N nylon membrane (Amerham UK). The blot was probed with the 700bp CFTR fragment corresponding to the *Xenopus* R-domain (clone pST101) which had been oligolabelled with ^{32}P-CTP to a specific activity of 10^9cts/min/μg (Pharmacia). The high stringency wash was for 30 mins with 0.2× SSC, 0.1%SDS at 65°C (31).

PCR amplification from cDNA

First strand cDNA was made from combined stage 44/48 total RNA using random oligonucleotides to prime cDNA synthesis at 42°C for 90mins with MuMLV reverse transcriptase (BRL) (31). PCR amplification of specific fragments was performed using 35 PCR cycles (each of 95°C for 1min, 55°C for 1min and 72°C for 1min) with 100ng of the specific primer and 500ng of degenerate primer (see Table 2 for primer details), 0.5μg reverse transcribed total RNA and 2U Taq XL polymerase (Northumbria Biologicals Ltd.) in 50μl total volume of PCR buffer (10× NBL PCR buffer is: 100mM Tris-HCl pH 8.8, 500mM KCl, 25mM $MgCl_2$, 1% non-ionic detergent) containing 0.1mM dNTP's. PCR products were resolved on 0.8% agarose gels and appropriate sized fragments excised and cloned into pBluescript vectors (Stratagene) prior to sequencing. A cryptic bacterial promoter creating cloning difficulties was found in exon 6 of the human CFTR gene (32). However, the −10 region corresponding to this 'promoter' is not conserved in *Xenopus*. The clones generated, and primers used, are illustrated in Figure 1 and Table 2 respectively.

DNA sequencing, alignment and sequence fidelity

DNA sequencing was by the chain termination method using double stranded DNA templates from the pBluescript plasmids. The sequence of both strands was determined using specific oligonucleotides and the Sequenase system (U.S. Biochemical Corporation). The nucleotide and amino acid sequence have been deposited in the GenBank™/EMBL Data Bank with the accession number X65256. DNA sequences were assembled and analysed using the INTELLIGENETICS programme and the University of Wisconsin Genetics Computer Group Software Package with minor adjustments to the alignments made by eye. As the clones from which the sequence was determined were generated by PCR, mutations may have been introduced during the amplifications. In order to reduce this possibility, high quality Taq polymerase was used (Cetus and NBL) and the number of amplification cycles kept to a minimum (typically 35). Sequence comparison of multiple clones from independent amplifications showed that PCR-induced errors were not a problem. We estimate the accuracy of the nucleotide sequence to be 99.9% based upon figures for the fidelity of AmpliTaq™ polymerase and the number of PCR cycles used (33). It should be noted, however, that as *Xenopus* is tetraploid each fragment could have been derived from any one of four alleles. Additionally, the seven residues at the amino and carboxyl ends of the protein are based upon the degenerate primers used for their amplification and these sequences cannot be stated unambiguously.

RNA based PCR

Samples (8μg) of total RNA from each of the *Xenopus* developmental stages 1, 6, 9, 14, 18, 23, 32, 40, 44 and 48, were reverse transcribed in duplicate (one set with reverse transcriptase [RT], the other without) in 20μl 1× NBL PCR buffer containing 1μg random hexamer primers, 400U MuMLVreverse transcriptase (BRL) and 1.25mM dNTP's at 42°C for 90mins. Samples were then boiled for 5 mins and chilled on ice before PCR analysis. 2.5μl (~ 1μg) of each sample was amplified by high stringency PCR using specific primers to the ODC gene and 30 PCR cycles each of 95°C for 1min, 60°C for 1min and 72°C for 1min. Analysis of the PCR products on an agarose gel showed bands of equal intensity in each lane for the '+' set (i.e. with RT) and no product in the '−' set (i.e. without RT) indicating approximately equal amounts of cDNA in the '+' set and no genomic DNA contamination in the '−' set (data not shown).

Similar amplifications were then repeated using specific primers to the *Xenopus* CFTR gene; XLR-5 (5'-CCGCAGGAGACAGTCTGTGC-3') and RXLR-7 (5'-GTACCAGAGGCAAACCTCTGA-3') which amplify a fragment of 542bp corresponding to bases 2285−2827 of the sequence. DNA fragments were resolved on a 1% agarose gel and Southern blotted onto Hybond-N (Amersham UK). The blot was probed with a 600bp probe, corresponding to clone pSTX (Table 2), using the Amersham ECL-gene detection system (Amersham UK) according to the manufacturers instructions.

RNAase protection assay

RNAase protection analysis was performed according to the methods of Isaacs *et al* (34) using a 240 nucleotide, ^{32}P-UTP labelled antisense RNA probe generated from clone pST101, with a modified RNAase digestion step; RNAase digestion was performed on 20μg total RNA from each stage for 20mins at 37°C using RNAase A (Boehringer Mannheim) at a concentration of 32μg/ml and RNAase T1 (BRL) at a concentration of 560units/ml. Control experiments showed that the band labelled CFTR was specific to the CFTR probe and not the result of cross hybridisation with the ODC probe. The products were resolved by electrophoresis on a 6% poly-acrylamide urea gel. Exposure times for the CFTR and and ODC probes were approximately 10−14 and 1−2 days respectively.

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