

Analysis of Alternatively Spliced Domains in Multimodular Gene Products - The Extracellular Matrix Glycoprotein Tenascin C

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1. Introduction

In 1977 it was discovered that the one-gene-one-enzyme hypothesis was not true (Chow et al., 1977; Berget et al., 1977). The primary transcription product can be spliced in different ways and give rise to several proteins depending on the exons being present in the final mRNA. This phenomenon is called alternative splicing and indeed is common to many genes. Several possible modes of alternative splicing are known and the most common one is the inclusion or exclusion of an exon, the exon skipping.

Based on polymerase chain reaction (PCR) techniques we developed a method to analyse combinations of alternatively spliced domains in multimodular gene products. This method was used to determine the combinatorial variability of tenascin C isoforms in the mouse central nervous system (Joester & Faissner, 1999) and in neural stem cells (von Holst et al., 2007).

Here, we present the method of amplifying different sized isoforms of a gene product with several alternatively spliced domains via PCR and the isolation and subcloning of the PCR products. Clones are analyzed for alternatively spliced domains contained therein by a dot blot *in vitro* hybridization method with domain-specific DNA probes which were generated using PCR.

2. Background information

Tenascin C is a multimodular glycoprotein of the extracellular matrix which is mainly expressed during central nervous system development and in pathological states such as brain tumours or lesions. We have studied the expression pattern of this molecule and its function *in vivo* and *in vitro* and collected evidence concerning its structural diversity. We and others determined its functions during neural development, in the adult neural stem cell niche and in lesions and tumours (Czopka et al., 2009, 2010; Dobbertin et al., 2010; Garcion et al., 2001, 2004; Garwood et al., 2011; Gates et al., 1995; Orend & Chiquet-Ehrismann, 2006; von Holst et al., 2007).

Tenascin C contains a constant part including eight constitutive fibronectin type III (fnIII) domains and a variable part of six alternatively spliced fnIII domains in the mouse which can be included independently into the gene product (figure 1).

The alternatively spliced fnIII domains of the tenascin C molecule have different functions, e.g. affecting the axon outgrowth of developing nerve cells or the migration potential of brain tumour cells (Rigato et al., 2002; Michele & Faissner, 2009; Broesicke & Faissner, personal communication). Therefore it is important to have a method to determine the isoform composition of the molecule in the tissue or cell cultures used.

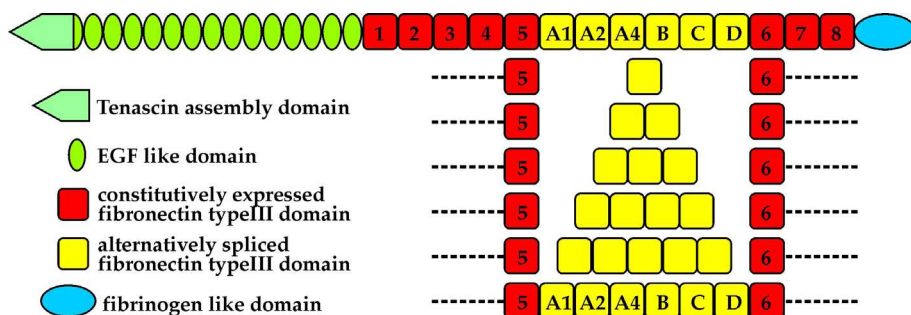


Fig. 1. Schematic representation of mouse tenascin C. The monomer consists of several distinct protein domains. At the N-terminal tenascin C assembly domain six monomers can be assembled to the so called hexabrachion (Erickson & Inglesias, 1984). 14,5 epidermal growth factor (EGF) like domains and eight constitutive fibronectin type III (fnIII) domains follow before the C-terminal globular lobe homologous to the beta- and gamma-chains of fibrinogen. Between the fifth and sixth constant fnIII domain up to six alternatively spliced domains can be inserted and an independent alternative splicing at each position could lead to the generation of 64 ($=2^6$) possible isoforms of the molecule. All possible numbers of domains can be inserted in the final splicing product, but the combination of cassettes is unclear in most cases. Only the largest variant necessarily contains all six alternatively spliced domains.

Gene products with different exons being alternatively spliced and inserted into the sequence can be distinguished by PCR when the sizes of the resulting mRNAs are different. A PCR analysis uses primers flanking the alternatively spliced region and results in amplicons with different sizes. These can be analysed by agarose gel electrophoresis and show bands in distinct positions. Tenascin C has six domains that can be alternatively spliced and independently inserted into the sequence. The analysis of these domains on an agarose gel shows the size of the resulting amplicons and therefore the number of inserted domains but leaves the question open which of the possible domains are included. A further analysis is therefore needed. We have shown that it can be performed using an *in vitro* dot blot hybridization technique to verify the exact domain combinations.

3. Analysis of isoform sizes in multimodular gene products by RT-PCR

When the sequence of the gene of interest is known primers can be generated which allow the amplification of the relevant region. The primers can either bind in the alternatively spliced region itself and therefore generate PCR products only when the target sequence is expressed. When the primers bind outside of the alternatively spliced part of the sequence the products can contain every possible insert additionally to the constant parts of the sequence which are defined by the primer binding sites. Additionally, isoforms without any insert can be amplified with these primer combinations.

Tenascin C has its variable region between the constantly expressed fnIII domains 5 and 6. We used two different primer combinations to determine the isoform pattern of the molecule in various tissues and cell cultures (figure 2). The primers 5s and 6as bind to the 5' end of the fifth and the 3' end of the sixth domain and result in PCR products with the smallest form containing only these two constant domains. Another primer pair we used was called 5for and 6rev and these bind to the 3' end of domain number 5 and the 5' end of domain number 6. The smallest amplicons are then represented by forms with one alternatively spliced fnIII domain. The further analysis was carried out with PCR products obtained with this primer pair.

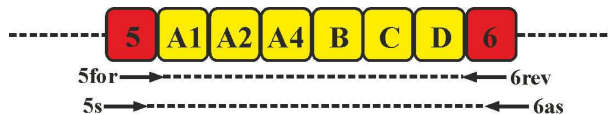


Fig. 2. Primer binding sites. Two different primer pairs were used to amplify the alternatively spliced region of tenascin C and analyse the expression profile of different isoforms. The primers 5s / 6as and 5for / 6rev bind to the constant fnIII domains 5 and 6 at their outer or inner tails, respectively. The primers 5s and 6as bind to the 5' end of the fifth and the 3' end of the sixth domain. The resulting amplicons therefore contain the minimum of two fnIII domains, namely 5 and 6. The further insertion of alternatively spliced cassettes increases the size of the PCR product. The primers 5for and 6rev bind to the 3' end of fnIII domain 5 and the 5' end of fnIII domain 6. Only PCR products with the minimum of one alternatively spliced fnIII domain can be generated. Every additional domain increases the size of the amplicons by 273 bp, the size of the single domains.

3.1 Expression analysis by RT-PCR

The expression analysis can be performed on RNA isolated from tissue or cell culture material which was processed by reverse transcription. Several commercially available kits help to isolate total RNA or mRNA from tissue or cell cultures. The resulting RNA can then be used to generate cDNA by reverse transcription which can also be carried out using kits from different suppliers. If oligo-dT primers or random primers are used for the reverse transcription makes no difference in our experience. The generated cDNA is the template for the PCR which possibly needs some optimization steps to generate all bands of interest. According to our experience it is of outstanding importance to test the performance of different Taq polymerases in advance because not every enzyme from each supplier will work equally efficiently. Different polymerases in their respective buffer system show variable results and should be adapted to the reaction requirements.

The PCR conditions with regard to annealing temperature and time, elongation time as well as concentration of cDNA, primers and Magnesium must be worked out in advance. Addition of DMSO or betain may be needed and checked when the standard conditions don't lead to the desired results. To be able to generate all the expected amplicons the longest product determines the elongation time. The rule of thumb to calculate 1 minute elongation time per 1000 base pairs gives a good estimation here.

The resulting PCR products can be processed on an agarose gel and the DNA bands made visible with ethidium bromide or a substitute. The concentration of the agarose must be high enough to discriminate between contiguous bands but sufficiently low that the longest products can enter the analysis area. A long gel chamber increases the migration way

and a lower voltage over a longer time period narrows the single bands and makes the discrimination easier.

We used brain tissue from postnatal mice or cultures of neural stem cells to isolate total RNA and analysed the expression pattern of the alternatively spliced forms of Tenascin C in the respective system (Joester & Faissner, 1999; von Holst et al., 2007). In these cases we found isoforms of all possible sizes to be present and performed the further analysis for isoforms containing between one and six additional cassettes. The use of the primer pair 5s and 6s leads to DNA bands on the agarose gel where the smallest one is 546bp, representing only the two constant fnIII domains 5 and 6 of 273bp each. Every additional cassette increases the amplicon size by 273bp. Therefore, we can see a "ladder" structure of up to seven DNA bands on the agarose gel when using this primer pair (figure 3B). When the primers 5for and 6rev are present in the PCR mix instead we get products where the smallest isoform contains the minimum of one alternatively spliced fnIII domain. The larger bands represent the larger forms with up to six fnIII domains. Here, we get the maximum of 6 DNA bands on the gel (figure 3A).

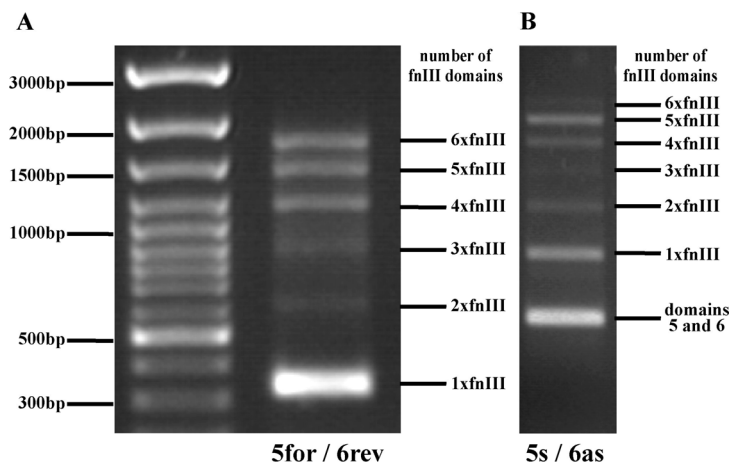


Fig. 3. Examples of tenascin C isoform PCRs. The primer pairs 5for / 6rev and 5s / 6as were used to amplify the alternatively spliced region of tenascin C. The PCR products were separated on an 1,2 % agarose gel. (A) The smallest amplicon generated using the primers 5for and 6rev contains only one of the alternatively spliced cassettes. The insertion of additional domains increases the product size by 273 bp. Up to six bands appear representing the different possible amplicon sizes. PCR products amplified with this primer pair were used for the further analysis of the domain expression profile after separation on an agarose gel. (B) The use of the alternative primer pair 5s and 6as leads to the generation of up to seven DNA bands on the agarose gel because the smallest band represents only the constant fnIII domains 5 and 6 without any insert. When alternatively spliced domains are included in the sequence the product size increases by 273 bp for each domain. Up to six domains can be added and therefore the largest DNA band on the gel represents the total of eight fnIII domains. This primer pair was mainly used for the analysis of expression profiles.

The agarose gel shows the expression profile of the alternatively spliced gene products in the analysed tissue or cells. The resulting amplicons answer the first questions in this respect: Are

different forms expressed in parallel? Are all possible product sizes present? What is the ratio between different forms? Does the expression profile change with the conditions?

3.2 Cloning of resulting PCR products

The analysis of the PCR products on an agarose gel answers the question for size and ratio of the isoforms expressed but leaves open which of the possible domains are contained in the bands. Some further experimental steps are necessary to determine the domains being expressed. Because several domain combinations can migrate in the same position they must be separated from each other. This can be achieved by subcloning the different PCR amplicons and analysis of the resulting clones.

The PCR bands are cut out of the gel under visual control at an UV desk and the gel slices collected in separate tubes. It is important to use different knives for each band because otherwise DNA from other bands might be carried over and contaminate the samples. Isolation of the DNA from the gel can be performed using classic methods or commercially available kits. The elution should be done with the minimal amount of water to avoid problems with following reaction steps. For the subcloning of the PCR products we used the TOPO-TA cloning kit from invitrogen but any other similar kit will do. In our experience it is important to handle the bacteria quite carefully and leave them grow in antibiotic-free medium for 30 minutes after the transformation. Spread the bacteria to LB agar plates with appropriate antibiotics then and let them grow over night.

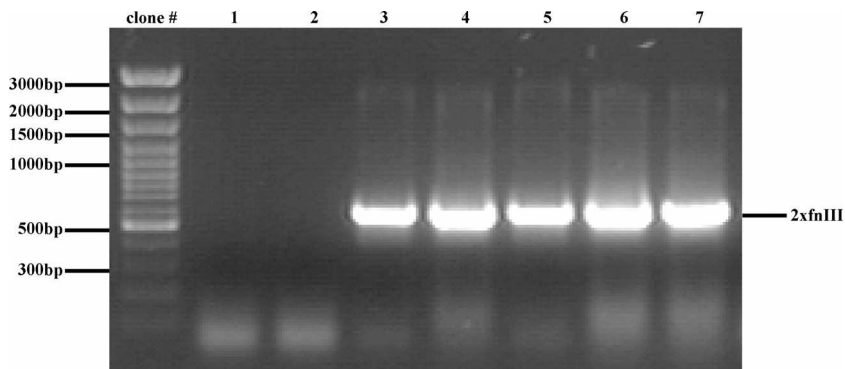


Fig. 4. Check for positive clones after direct colony lysis. The colonies grown after the cloning and transformation of the PCR products are checked for their content of fnIII domains. The primer pair 5for / 6rev was used to generate amplicons of the expected size when the clones have taken up the plasmids containing the fnIII domains. This example shows seven clones from a DNA band containing 2 fnIII domains. Two of the clones shown here do not contain any fnIII domains and are therefore not selected for the following screen. The other clones show PCR bands of the expected size and are analysed in the subsequent dot blot hybridizations.

The content of the resulting clones can quickly and easily be checked by direct lysis of the bacteria and a subsequent PCR with the primers used before. The colonies grown on the agar plate are picked with a pipette tip and transferred to another (the "master" plate) into numbered fields. The tip is then shaken in 25 μ L 70% ethanol in PCR tubes to lyse the bacteria. The master plate can be placed in the incubator while in the meantime the colonies are checked for their content. In an incubation step the ethanol is evaporated at 80°C for approx. 15

minutes before the PCR master mix containing buffer, primers and polymerase is added. The reaction conditions can be the same as before. The products can be analysed on an agarose gel and should show single bands in the expected position for each positive clone (figure 4). These can subsequently be picked from the master plate and propagated in miniprep scale. The plasmid DNA from the miniprep cultures can be isolated by alkaline lysis or with appropriate kits.

3.3 Analysis of clones - dot blot

Of course, these plasmids could be sequenced and their composition clarified by this method at this point. Because sequencing is not cheap when analysing hundreds of clones a method was developed that renders the identification of many samples in one step possible and is cheaper. The basis is a dot blot of the isolated plasmids to nylon membranes which subsequently can be used in hybridizations with domain specific probes.

The plasmid solutions should be adjusted to similar concentrations with water to have equal amounts of target DNA in the spots. The easiest way to apply the plasmids to the membranes is the use of a dot blot apparatus with a vacuum manifold, but it is also possible to spot the liquid using a master plate onto the membrane which is placed on filter paper. For our analysis we used Hybond N+ membranes from Amersham which were pre-wetted with 10x SSC (1,5 M NaCl; 150 mM Na₃Citrate, pH 7,0) buffer. Because there are six possible domains to detect (A1, A2, A4, B, C and D) and we used a negative control we prepared seven membranes with identical spot patterns. The plasmid solutions were diluted in 10x SSC in a volume of 100 μ L when we used the dot blot apparatus and 6 μ L when a pattern was used.

After application of the plasmid solutions the membranes are incubated for 10 minutes in denaturing buffer (500 mM NaOH; 1,5 M NaCl) and 10 minutes in renaturing buffer (500 mM Tris/HCl, pH 7,5; 1,5 M NaCl) to prepare the DNA for the hybridization. The nylon membranes are dried and baked at 80°C for two hours to have the DNA bound covalently to them. These dot blots can be stored for some time at room temperature.

3.4 Positive and negative controls

To determine the specificity of the method and to be sure that no false-positive or false-negative results appear the use of positive and negative controls is important. For every application appropriate controls must be defined. In our case we could exploit the fact that the fnIII domain number 6 is not included in the alternatively spliced region which we amplify with the primer pair 5for / 6rev in the initial PCRs. Therefore a probe detecting the fnIII domain 6 serves as negative control. Another control we use is a plasmid, called pJT1# which contains the constant part of tenascin C between the fnIII domains 2 and 8, but none of the alternatively spliced domains. The positive control is a plasmid containing all six alternatively spliced domains. On the dot blot it is applied in addition to the clones under investigation.

3.5 Generation of domain specific DNA probes by PCR

The hybridization of membrane-bound DNA with probes detecting defined DNA fragments identifies specific sequences in the bound nucleic acids. Probes detecting the desired target sequences are generated based on the cDNA of these fragments which are cloned into common plasmid vectors. We used the sequences of the tenascin C fnIII domains A1, A2, A4, B, C, D and 6 as negative control which were inserted in pBluescript II KS+ vectors.

These inserts are labelled to use them in expression studies. The labelling with fluorescein has several advantages. When using non-radioactive probes no special safety regulations must be obeyed. Additionally, the probes can be used for a longer time period. This is of special advantage when several probes are used in parallel. Manufacturer's data state that fluorescein-labelled probes are stable for 6 months without decreasing activity. Radioactively labelled probes would lose sensitivity after a few days because the isotopes disintegrate continuously. Indeed, the probes generated in our lab could be used for several years (Joester & Faissner, 1999; von Holst et al., 2007).

The labelling was performed with fluorescein-coupled dUTP (Amersham). The manufacturer's labelling kit could not be used because it is based on a random primer labelling method. This uses the hybridization of 8 to 10 bases long random primers to single DNA strands. In a polymerization mix with the labelled nucleotide the Klenow fragment of the DNA polymerase I generates the complementary probe. But the tenascin C domains are less than 300 base pairs long and therefore have only a few potential binding sites for random primers which may lead to only very short probes. The labelling efficiency is too low (1 labelled base in 50 bases, according to manufacturer's data) to achieve an appropriate labelling frequency. Therefore we developed a labelling protocol which uses a PCR method to generate dUTP-labelled DNA probes.

The Taq polymerase incorporates dUTP with less efficiency than unlabelled dTTP. Therefore the exclusive use of dUTP would show the optimum of labelled probe but only low yield of amplification product. A low amount of fluorescein-dUTP and higher amount of dTTP reverses this effect and leads to a higher product yield but low labelling efficiency. We adjusted the PCR conditions to the optimal yield of labelled amplification product.

The optimal reaction conditions required only 10 pg of plasmid DNA and a low amount of dNTPs (20 μ M). The reaction mix contained 60 mM Tris/HCl, pH 8,5; 15 mM $(\text{NH}_4)_2\text{SO}_4$; 2 mM MgCl_2 ; 0,2 μ M sense primer; 0,2 μ M antisense primer and 1 Unit Taq polymerase in 25 μ L volume. The cycling conditions are dependent on the hybridization temperature of the respective primers and the length of the expected product.

In the first labelling reactions with domain C different amounts of dTTP were replaced with FI-dUTP (3 to 50% equivalent to 0,6 to 10 μ M FI-dUTP). The amount of the products increases with decreasing amounts of the labelled nucleotide. The labelling efficiency was also tested on dot blots with different concentrations of the plasmid containing the C domain. 1 μ L of the PCR products were used in the hybridization solution. After an over-night incubation the blots were developed with an alkaline phosphatase-coupled antibody detecting fluorescein. The detection sensitivity was proportional to the concentration of the FI-dUTP used in the labelling reaction. The subsequent labelling reactions were performed using 17,5 μ M dTTP and 2,5 μ M fluorescein-11-dUTP. All probes detecting the fnIII domains A1, A2, A4, B, C and D of tenascin C were labelled with this method and called FI-A1, FI-A2,... Figure 5 shows the resulting PCR amplicons.

The fluorescein-labelled probes were tested for their detection capability of different dilutions of the respective plasmids. The sensitivity was different for the probes and therefore their concentration was adjusted in the hybridization solution. The hybridization results show that the sensitivity is equal between 3 pg up to 1 ng of the target sequence (figure 6A). This sensitivity is much higher than that seen for agarose gels stained with ethidium bromide which is in the range of 1 ng DNA. The sensitivity was tested regularly to adjust the stability or labelling efficiency of the different probes but none showed a significant reduction in detection efficiency over time.

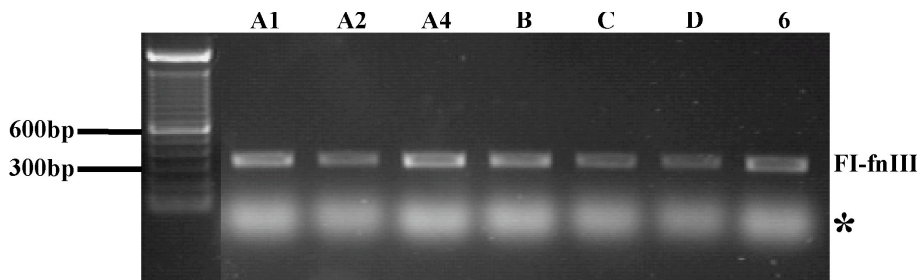


Fig. 5. Fluorescein-labelled probes on an agarose gel. The DNA probes labelled with FI-dUTP were applied to an agarose gel and show bands in the expected size of less than 300 bp. The asterisk designates the fluorescence signal of the non-incorporated nucleotides.

The specificity of the fluorescein-labelled probes was tested with seven dot blot stripes containing the plasmids pA1, pA2, pA4, pB, pC, pD, p6 and pJT1# in equal concentrations. The stripes were hybridized with the probes for the single fnIII domains (FI-A1, FI-A2,...) and the alkaline phosphatase reaction was developed (figure 6B). Highly stringent hybridization and washing conditions minimised the cross-reactivity of the probes with unspecific target sequences. These conditions included the hybridization and the first washing steps at 72°C

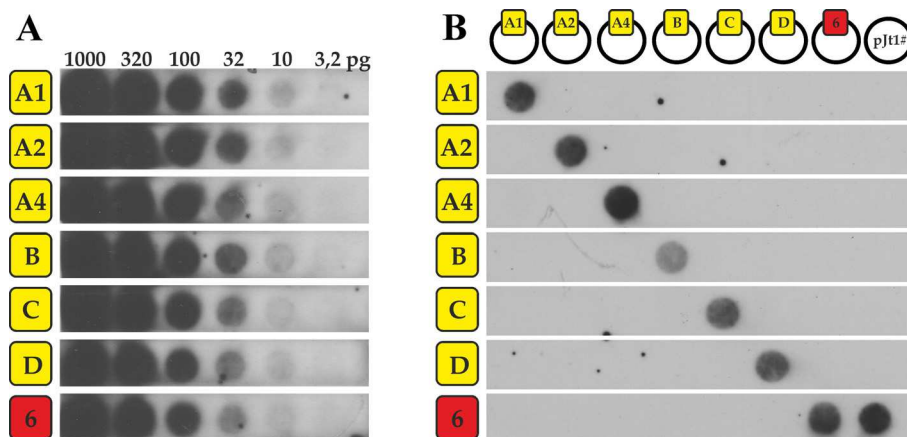


Fig. 6. (A) Sensitivity of fluorescein-labelled probes. The plasmids containing the single fnIII domains A1, A2, A4, B, C, D and 6 (designated pA1, pA2, ...) were diluted and applied to the nylon membranes in dots. The membranes were incubated with the respective probes and the reaction developed with an anti-fluorescein antibody coupled to alkaline phosphatase. A minimum of about 10 pg of target sequence was detected by each of the probes. The probes were diluted so that all of them detected their targets in a comparable way. (B) Specificity of fluorescein-labelled probes. Seven identical dot blots containing 10 ng of the plasmids pA1, pA2, pA4, pB, pC, pD, p6 and 19 ng pJT1# (corresponding to 1 ng target sequence) were hybridized with the different fluorescein-labelled probes. The probes detect their target sequences with high specificity. Although domains A1 and A4 are highly identical the probes do not show a significant cross-reactivity. The probe FI-6 detects the plasmid pJT1# which contains domain number 6.

and the use of 0,5% SDS in the washing buffer. The highest probability for a cross-reactivity exists between the domains A1 and A4 because their nucleotide sequence is 80% identical. Only in a few cases a light background signal could be detected when using these probes.

3.6 Hybridization

The generated probes are used in an *in vitro* hybridization protocol and applied to the nylon membranes containing the plasmid DNA from the clones which shall be analysed. The nylon membranes with the bound plasmids are washed in 5xSSC and pre-incubated in hybridization solution (5x SSC; 0,1% SDS; 5% dextrane sulfate; 5% liquid block (Amersham)) for 30 minutes at 72°C with gentle agitation. An appropriate amount of the probes which must be determined in preliminary experiments is added to 200µL of hybridization solution. The probes are denatured at 96°C for 5 minutes and applied to the membranes. The hybridization takes place over night at 72°C with constant agitation in a hybridization oven.

3.7 Signal detection

We used two different methods for the detection of the hybridized probes. A detection protocol to obtain chemoluminescence signals uses the fluorescein gene images CDP-*Star* detection system (Amersham). The other option was the development of a colour reaction using NBT and BCIP as alkaline phosphatase substrates.

When the DNA on the membranes was hybridized over night with the fluorescein-coupled probes the membranes can be washed for 2 x 15 minutes in wash buffer 1 (0,1x SSC; 0,5% SDS) at 72°C. To block unspecific binding sites they are incubated for one hour in blocking buffer (10% liquid block (Amersham) in detection buffer (100 mM Tris/HCl, pH 7,5; 300 mM NaCl)) before the alkaline phosphatase-coupled anti-fluorescein antibody (1:5000 in detection buffer with 0,5% BSA) is applied for an hour. Unbound antibody is washed away with wash buffer 2 (0,3 % Tween-20 in detection buffer) 4x 8 minutes. For the chemoluminescence detection the blot is moistened with a diioxetane-based substrate solution (CDP-*Star* detection reagent (Amersham)). After 3 minutes the excess substrate solution is removed and the blot placed between two sheets of foil and laid on an autoradiographic film. Depending on the DNA concentration the optimal detection time was between 10 and 60 minutes.

For the alternative developing method the membranes are washed 3x 5 minutes in wash buffer A (100 mM Tris/HCl, pH 7,4; 150 mM NaCl; 0,3% Tween-20) after the antibody incubation, 2x 5 minutes in wash buffer B (100 mM Tris/HCl, pH 9,5; 100 mM NaCl) and 3x 10 minutes in TBS (50 mM Tris/HCl, pH 7,5; 150 mM NaCl). To develop the colour reaction the membranes are wetted with staining solution containing NBT and BCIP (Roche) and not shaken any more. The colour reaction will appear after five to 60 minutes. The reaction can be stopped with water and the membranes dried afterwards. Because the detection sensitivity is lower for the colour reaction a higher amount of plasmid DNA must be used in this case. 50 ng of target sequence in the spots lead to good results (data not shown).

3.8 Analysis of domain combinations

The read-out of the results is straight forward. The membranes show positive signals whenever the respective domain is present in the clone. Every plasmid DNA shows a specific pattern of positive and negative signals and therefore stands for the presence or absence of a given single domain.

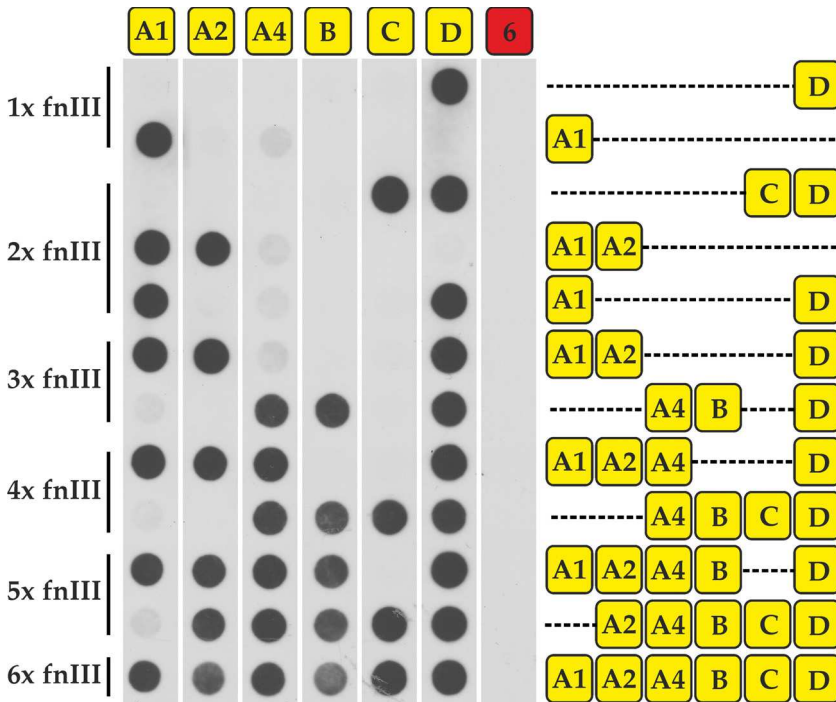


Fig. 7. Example of screening results. Plasmids containing different numbers of alternatively spliced fnIII domains were applied to nylon membranes in dots. Seven identical blots were generated and hybridized with the fluorescein-labelled probes FI-A1, FI-A2, FI-A4, FI-B, FI-C, FI-D and FI-6 as negative control. After the development of the alkaline phosphatase reaction the dot blots show positive signals whenever the respective fnIII domain is present in the plasmid. Therefore the domain combination of every single clone can be directly read out from the blots.

In intensive studies of the expression pattern of Tenascin C isoforms in the developing brain and in neural stem cells (Joester & Faissner, 1999 and von Holst et al., 2007) we detected 28 different isoforms of Tenascin C out of 64 possible ones which could theoretically be generated with six independently spliced domains ($=2^6$). We had several hundred clones to identify which contained between one and six alternatively spliced fnIII domains. The membranes we prepared were handled separately depending on the expected number of domains to be present in the plasmids. Figure 7 shows an example of the analysis of several clones with different numbers of fnIII domains. Plasmids from the distinct subcloning reactions were spotted onto seven nylon membranes and hybridized with the probes FI-A1, FI-A2, FI-A4, FI-B, FI-C, FI-D and FI-6 as negative control. The signals show that different combinations of fnIII domains can be contained in the plasmids. The clones with only one alternatively spliced domain displayed here for example contain the domains A1 or D, respectively. Indeed, these were the most common domains among single-domain clones when a complete screen was performed (Joester & Faissner, 1999; von Holst et al., 2007). The variability of domain combinations is higher in the middle-size clones with two, three or four alternatively spliced fnIII domains. The plasmids containing five additional cassettes on the other hand show

usually the absence of fnIII domain C and only few miss A1. The extensive screens for the expression profiles of fnIII domains being expressed in postnatal mouse cerebellum or neural stem cells show that the possible variability among the clones is not utilised. 64 different isoforms of tenascin C would be theoretically possible but only 28 forms were found. Some combinations of domains were never seen like the direct link between the fnIII domains C and 6 or A4 and C.

3.9 PCR of single domains

To confirm the results of the hybridization and to clarify ambiguous signals we carried out PCRs for the single fnIII domains that were detected in the plasmid DNA. It is important to highly dilute the plasmid DNA and use only 10 to 20 pg plasmid DNA as template and to use highly specific PCR conditions. The specificity of the PCR conditions was confirmed before because the fnIII domains show high similarities and could therefore lead to false-positive signals when using standard PCR conditions on plasmid templates. We used a 2-step PCR with a high annealing temperature of the primers of 72°C and combined the annealing step with the elongation step to a 40-seconds 72°C incubation step. The cycling was therefore between 20 seconds 94°C and 40 seconds 72°C. We also skipped the final 5-minutes elongation step which we usually applied, especially for the addition of adenosomes for cloning purposes.

Figure 8 shows the high specificity of these PCR conditions when the different fnIII domain-containing plasmids were used in these reactions. Only for those plasmids amplicons were generated when the respective primer pair was used. Therefore, we had an additional tool to confirm the dot blot results.

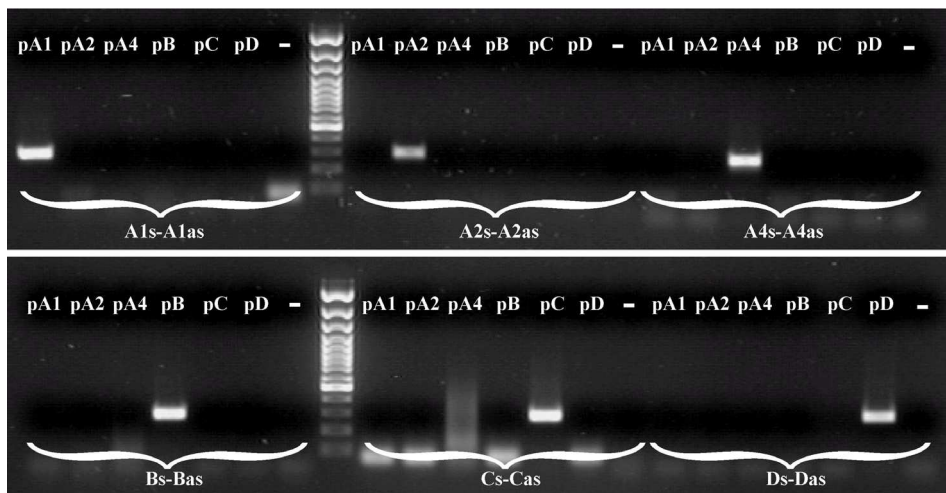


Fig. 8. PCRs for single fnIII domains. The primer pairs A1-s / A1-as, A2-s / A2-as,... were used in PCRs for the amplification of single fnIII domains. The domains have some similarities in their sequences. Therefore the PCR conditions must be highly specific. PCRs with stringent conditions amplify only products from plasmids containing the respective domain. Such conditions can be used to test plasmids with unclear domain composition.

4. Adaptation to general application

Many genes are subject to alternative splicing and most of them show an exon skipping mode which implies the inclusion or exclusion of single exons. When the possible exon structure leading to the appearance or absence of single domains is known the expression profile of

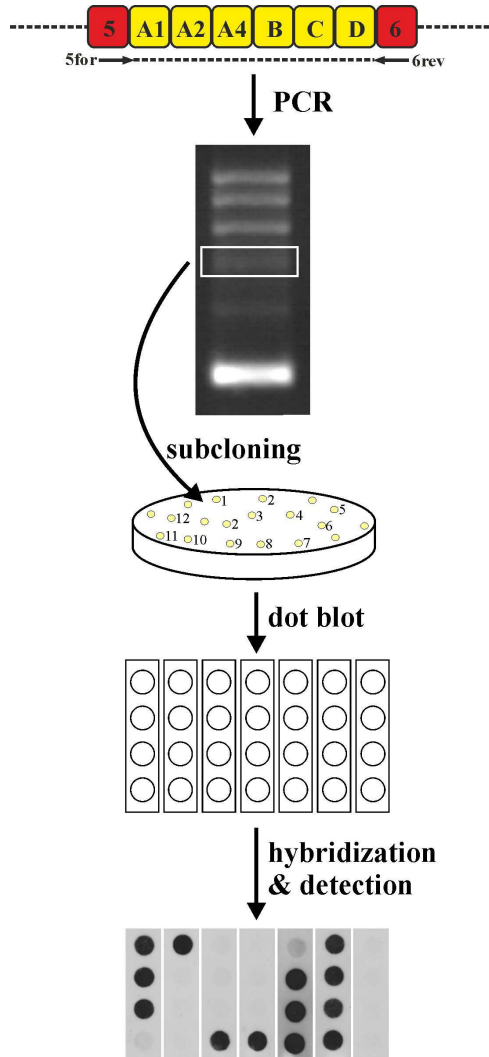


Fig. 9. Schematic presentation of the method. Primers flanking the alternatively spliced region of the molecule are used to generate PCR products of different sizes which are separated on an agarose gel. The single bands are cut out off the gel and subcloned separately. The resulting clones can be analysed with a dot blot hybridization procedure with non-radioactively labelled probes. Positive and negative signals display the domain composition of every single clone.

the domains can be analysed using the method presented here. Some preliminary steps are necessary before a screen for expressed domains can be started but when the system is set up once it can be used for the screening of many PCR products over a long time.

To start such a screen the following steps must be accomplished:

1. Clarify the domain sequence
2. Generate primers detecting the single domains
3. Clone the single domains into plasmid vectors
4. Use these vectors as templates for PCRs generating dUTP-labelled DNA probes

A screen includes the generation of the plasmids and the dot blot before the hybridization can start. Therefore conduct the following steps to start a screen:

1. Isolate RNA from the tissue or cell type under investigation
2. Prepare cDNA based on this RNA
3. Use this cDNA as template in PCRs for the alternatively spliced region of your gene
4. Separate the amplicons on an agarose gel and cut off the single bands
5. Clone the PCR products into plasmid vectors
6. Dilute the plasmid vectors to appropriate concentrations
7. Apply the plasmid solutions onto nylon membranes
8. Denature the DNA and bind it covalently to the membranes
9. Hybridize the DNA on the membranes with the probes
10. Wash under stringent conditions
11. Apply an antibody to the labelling marker
12. Develop the enzyme reaction
13. Read out your domain structure

Figure 9 shows the summary of the method:

5. Conclusion

With the method presented here we developed a possibility to unravel unknown structures of splice products for alternatively spliced transcripts. The example we analysed was the extracellular matrix molecule tenascin C but any other multimodular protein can be examined in a similar way. With some preliminary preparations an operational tool is at hand which makes the screening of many clones and therefore the generation of an expression profile possible.

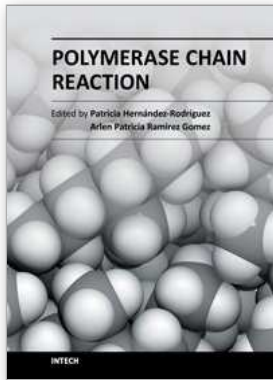
6. Acknowledgements

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