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An evidence of presence of DNA-binding proteins in selection of dystrophin gene promoter

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Abstract

The expression of dystrophin gene has been found to be regulated through different promoters in different tissues. To understand the mechanism of tissue specific selection of promoter DNA-protein interaction studies were carried out using tissue specific nuclear extracts. Several tissue specific binding proteins have been detected which could have their role in selection and maintenance of tissue specificity of promoters.

Keywords: DNA-binding proteins, dystrophin, promoter

Introduction

Dystrophin gene, which is altered in Duchenne and Becker muscular dystrophies, expresses differently in different tissues. The difference has been attributed to the presence of several different promoters in muscle and non-muscle tissues. At present six initiation sites are known, for each of which separate promoter has been identified. Three of these promoters are specific for brain, muscle and Purkinje cells and located in the 5' region of the gene. Another promoter, detected in lymphoblastoid cells, is located >500 kb upstream to brain promoter (Nudel *et al.*, 1988; Boyce *et al.*, 1990; Nishio *et al.*, 1994).

Structural studies of brain, muscle and Purkinje cell type promoter have been identified having several *cis*-acting sequences like E box, CCArGG box and MEF2 like sequences (Klamut *et al.*, 1990; Gilgenkrantz *et al.*, 1992; Muntoni *et al.*, 1995). The promoter found in lymphoblastoid cells also contain MEF2 like elements and TATA box.

The mechanism which regulate the selection of different promoter in different tissues is not well understood. Therefore, we have planned to look for the presence of tissue specific DNA binding proteins, which may have some role in selection of dystrophin promoters in different tissues using gel retardation and Southwestern assays.

Materials and Methods

Preparation of probe

Cloned muscle and brain promoter probes from human origin (gift from Dr. F. Boyce, Boston, U.S.A.) was amplified in *E. coli* DH5α and plasmids were extracted using alkali lysis method (Sambrook *et al.*, 1989). After digestion with restriction enzyme, purified promoter sequences were labelled with [α-^{32}P]ATP at 3' end for gel retardation assay and by random priming for Southwestern hybridization (Garabedian *et al.*, 1992). Probes were purified by agarose gel electrophoresis followed by glass bead binding (Vogelstein and Gillspie, 1979).

Preparation of nuclear extract

Nuclear extract from rat brain, heart, lung and skeletal muscle were prepared according to the method of Gorski *et al.* (1986), except that isolated proteins were dialyzed in microdialysis system (BRL Life Technologies Inc., MD, U.S.A.) overnight at 4°C. After dialysis, protein precipitate was removed by centrifugation and the clear supernatant was aliquoted, snap frozen in liquid nitrogen and stored at -70°C. Protein concentration was estimated by Bradford dye binding assay (Bradford, 1976)

Gel retardation assay

Six to ten μg of nuclear extract was incubated with 0.5 μg of denatured *E. coli* DNA in the presence of binding buffer (25 mM Tris, 0.66 mM EDTA, 40 mM KCl, 6 mM $MgCl_2$, 0.3 mM $ZnCl_2$, 0.5 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 10% glycerol, pH 7.9), in ice for 10 min. 1-5 ng of muscle promoter probe (20,000 c.p.m.) was added and mixture was again incubated in ice for 20 min. Samples were loaded on 4% polyacrylamide gel and electrophoresed at 10 V/cm for 2 h using buffer containing 0.25 mM Tris, 0.4 mM glycine, 2 mM EDTA (pH 8.5). The gel was dried and exposed for autoradiography.

Western blotting

Ten μg of nuclear extracts from each tissue were boiled for 90 s with sample loading buffer (3% SDS, 62.5 mM

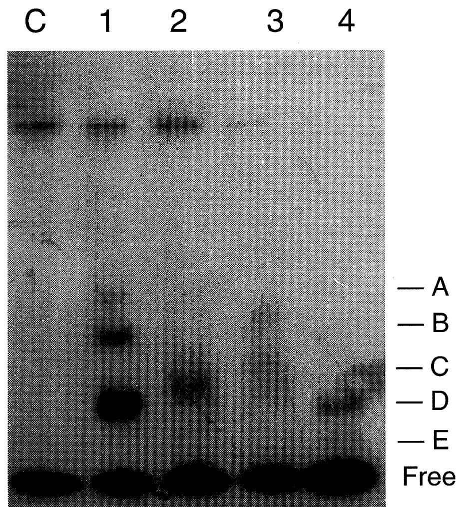

Figure 1. Gel retardation assay with muscle specific promoter. Binding of the 3' end labelled promoter to nuclear extract from different tissues of adult rat. C, control (absence of nuclear extract); 1, brain; 2, heart; 3, lung; 4, muscle; Free, unbound labelled promoter.

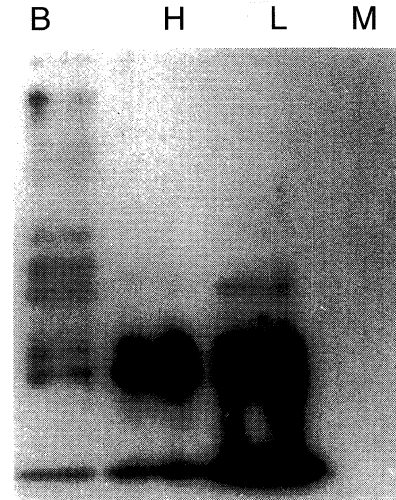

Figure 2. Southwestern hybridization of nuclear extract from different tissues of adult rat with muscle specific promoter on nitrocellulose membrane. B, brain; H, heart; L, lung; M, muscle.

Tris-Cl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol and 0.2% bromophenol blue) and applied to 7.5% SDS-polyacrylamide gel and electrophoresed for 2 h at 65 V in buffer containing 25 mM Tris and 192 mM glycine. The protein was transferred to nitrocellulose membrane using transfer buffer (50 mM Tris, 380 mM glycine, 0.1% SDS and 20% methanol) in Western blot sandwitch (Tefco, Japan).

Southwestern hybridization

Nitrocellulose membrane containing nuclear extracts was placed in a plastic dish and large volume of Z buffer (25 mM Hepes, pH 7.6, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% NP-40, 0.1 M KCl, 10 μM $ZnSO_4$, 1 mM dithiothreitol) with 6 M guanidium chloride, was added to it to cover the membrane and incubated with gentle shaking for 10 min. This step was repeated with an equal volume of the same buffer. This solution was decanted into a measuring cylinder and mixed with an equal volume of Z buffer (without guanidium chloride). Half of this solution was added to the filter and incubated for 10 min with gentle shaking. This step was repeated five more times, which decreases the guanidium chloride concentration to 0.1 M. Membrane was washed twice with Z buffer (lacking guanidium chloride) for 5 min each. The membrane was transferred to a fresh dish and incubated for 30 min in blocking buffer (Z buffer containing 3% non-fat dried milk and ^{32}P-labelled double strand promoter sequence) and incubated for 30 min. Buffer was carefully decanted into radioactive waste and the membrane was washed three times in Z buffer for a total time of 15 min. After washing, membrane was placed between two pieces of cling film and exposed to X-ray film.

Results

Gel retardation assay

Gel retardation assay of the nuclear extract from brain, lung, heart and skeletal muscle with muscle type promoter of dystrophin gene showed presence of 5 different DNA protein complexes (Figure 1). Band A was observed only in brain, band B in brain and lung both while band C is visualized in heart and lung. Brain and muscle showed presence of another complex (band D). Band E was detected only in muscle tissue. The specificity of complexes were confirmed by inhibition of binding with unlabelled promoter sequences.

Southwestern assy

Southwestern hybridization assay detected several complexes with muscle specific promoter. At least 13 complexes with brain, 4 with heart and 3 were detected with lung nuclear extract. Due to the unstability of muscle nuclear protein on nitrocellulose membrane, no complex could be detected specific for this tissue (Figure 2). Experiments carried out using immobilon-P membrane showed stability of muscle nuclear protein but no complex was observed with this protein (data

Figure 3. Southwestern hybridization of nuclear extract from different tissues of adult rat with brain specific promoter. B, brain; H, heart; L, lung; M, muscle.

not shown).

Experiments using brain specific promoter detected 5 complexes each with brain and lung, 2 with heart, and only one band with skeletal muscle nuclear extract (Figure 3).

The specificity of bands were checked by first adding unlabelled probe and then labelled probe during hybridization. No band was detected in any of these experiments.

Discussion

Understanding the mechanism of the cell type specific gene expression is a fundamental problem in molecular biology. The studies related to muscle differentiation has provided some insight into the field because induction of several independent muscle specific genes are required for the specialized function of muscle fibres. Dystrophin, a sarcolemmal protein, is required for the integrity of plasma membrane during muscle contraction. The gene responsible for this protein expresses differently through different promoters depending upon cell type. These promoters are involved in control of tissue and developmental specificities of its expression (Nudel *et al.*, 1989; Bies *et al.*, 1992).

Dystrophin gene, which primarily expresses in muscle tissues also found to express in lung, liver, brain and several other tissues (Chelly *et al.*, 1988). *Cis*-acting sequences identified in the promoter of dystrophin gene (E box, CCArGG box and MEF2 sites) binds to regulatory factors like Myo D1, serum responsive factor (SRF), muscle enhancing factors (MEF-2) and RSRFs (related to SRFs). These proteins confer muscle specificity and are capable of inducing myogenesis in non-muscle cells. Low expression of dystrophin gene in non-muscle tissues may be accounted due to non availability of these regulatory factors. It looks like that in these tissues certain other tissue specific factors trigger dystrophin gene expression in combination with these myogenic factors.

The functioning of different promoters in neural and glial cells (Chelly *et al.*, 1990) seems to be controlled by brain specific binding factors (Figures 1, 2, 3). Similarly enhanced expression of dystrophin gene in muscle (Chelly *et al.*, 1988) may be accounted due to muscle specific factor observed in gel retardation assay (Figure 1).

Interaction of various positive and negative regulatory elements play an important role in expression of dystrophin gene. Potential *cis*-acting sequence identified in dystrophin promoter are MEF2 binding site, MCAT site, E boxes and CCArGG box with some negative domains (Gilgenkrantz *et al.*, 1992). The binding proteins which we have observed might be interacting with these sequences and regulating dystrophin gene expression. It is possible to reason that the binding proteins observed from heart does not play an important role in selection of promoter because the heart tissue is reported to have dystrophin mRNA through both type of promoters (Bies *et al.*, 1992), which indicates that binding proteins present in heart are important only in regulation of dystrophin gene rather than in selection of promoters. Similarly, binding proteins observed in lung nuclear extract may also have their role in selection of brain or muscle type of promoter because we could not observe any correlation of these complexes with muscle or brain.

We, for the first time show an evidence of involvement of various binding factors in tissue specific selection of promoter for dystrophin gene expression. A detailed analysis of sequences present in promoter and characterization of binding proteins involved in interaction with these, is required to completely understand the regulatory mechanism of dystrophin gene expression.

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