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Trans-activation of the murine dystrophin gene in human–mouse hybrid myotubes

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Myotube cultures of the myogenic cell line, C2, produce significantly lower levels of dystrophin than primary mouse cultures. We demonstrate that expression of the C2 dystrophin gene increases 10-fold in hybrid myotubes formed by fusion of C2 and dystrophin-deficient human myoblasts from a Duchenne muscular dystrophy patient. These results indicate that C2 cells are deficient in endogenous gene regulatory factors which enhance dystrophin expression, and that the C2 cell line may therefore be used to identify putative trans-acting factors involved in the regulation of dystrophin gene expression.

Dystrophin; C2 cell line; Hybrid myotube; Gene regulation

1. INTRODUCTION

Dystrophin is a 427 kDa protein product of the Duchenne muscular dystrophy (DMD) gene [1]. It is expressed in a tissue-specific and developmentally regulated manner, being found most abundantly in terminally differentiated myogenic cells of all types [2].

A region 850 bp upstream of the cap site of the gene was initially shown to be capable of regulating muscle-specific transcription of a reporter gene in cell culture [3]. In particular a 96 bp sequence containing conserved CArG-box and E-box motifs was found to be sufficient to confer partial muscle specificity, but other potential positive and negative regulatory elements were detected upstream [4]. The CArG-box around position −91 bp has activating effects in muscle, cells and is clearly involved in regulation. No influence of MyoD1 however, on the proximal E-box (−58 bp) was found, consistent with the pattern of expression of the DMD gene not only in skeletal muscle, but also in cardiac and smooth muscle where MyoD1 has not been found [5–7].

An interesting observation made by Klamut et al. [3] was a deficiency in the expression of dystrophin mRNA, in myotube cultures of the murine myoblast cell line, C2, in comparison to cultures of primary myoblasts. The dystrophin promoter–reporter gene constructs, while being highly expressed in primary myotube cultures, were only very weakly active in C2 cultures. This suggests that deficiency of dystrophin expression by C2 cells may result from abnormal regulation of gene expression.

In this report we present evidence which shows that C2 myotube cultures do produce full-length dystrophin, but at levels which are 10-fold lower than those of primary myotube cultures. Furthermore we show that the expression of the C2 dystrophin gene can be increased greatly in hybrid myotubes formed by co-fusion of C2 and primary myoblasts. These results indicate that C2 cells are deficient in trans-acting gene regulatory factors which have a positive effect on dystrophin expression. C2 cells exhibit normal regulated expression of both skeletal-actin [8] and creatine kinase genes [9], indicating that other myogenic regulatory factors are generally present and functionally active. We therefore suggest that the C2 cell line can provide a model for the identification of novel factors which influence dystrophin gene expression in a relatively specific manner.

2. MATERIALS AND METHODS

2.1. *Preparation of muscle cultures*

Normal human and mouse myoblasts were obtained by primary enzymatic dissociation [10] of limb muscle from human foetal abortis tissue, 16–18 weeks gestation, and 3-week-old mice. Dystrophin-deficient primary human myoblasts were grown from a biopsy of a DMD patient, in which a dystrophin gene deletion spanning exons 49–50 had been demonstrated. C2C12 cells are a subclone of the C2 cell line isolated from normal mouse muscle [11,12]. Myoblasts were seeded at 4×10^4 cells/cm^2 on collagen-coated plastic tissue culture dishes and glass coverslips. Cultures were allowed to proliferate in Ham's F10 nutrient medium, with 15% v/v FCS, 2 mM glutamine, insulin at 100 μg/ml, 1 μM dexamethasone, fibroblast growth factor and epidermal growth factor each at 10 μg/ml. When confluent, cultures were transferred into Dulbecco's modified Eagles medium with 5% v/v heat-inactivated horse serum and 2 mM glutamine, to facilitate terminal differentiation. Hybrid myotube cultures were constructed by seeding

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C2C12 cells (10^4/cm^2) onto a confluent monolayer of human DMD myoblasts before transferring into differentiation medium.

2.2. *Immunocytochemistry and Western blot analysis*

Dystrophin expression in 14-day-old myotube cultures was examined by immunocytochemical and Western blot analysis. Cells grown on coverslips were immunostained, as described by Dickson et al. [13] with a mouse monoclonal antibody to dystrophin called Mandys 1 [14]. Human–mouse co-cultures were double-stained with Mandys 1 and Hoechst dye #33258 [11] to identify hybrid myotubes by differential staining of human and mouse nuclei. Stained cells were viewed on a Zeiss microscope equipped with phase contrast and epifluorescence optics. For Western blot analysis myotube cultures on 100 mm plastic tissue culture dishes were harvested in 0.5 ml SDS-sample buffer, sonicated for 20 s and solubilised by heating at 100°C for 4 min. Equivalent aliquots were electrophoresed on 6% polyacrylamide minigels, blotted and probed with a mouse mononclonal antibody to dystrophin, 6D3 [15] as described previously [16]. Quantitative analysis of dystrophin bands revealed by Western analyses was performed by measuring absorption of white light on a Shimadazu scanning densitometer.

3. RESULTS

At the mRNA level previous studies have suggested abnormally low levels of dystrophin gene expression in C2 myotubes. To confirm this observation at the protein level we used antibodies to dystrophin to perform immunofluoresence stains on C2 cultures. A positive control for dystrophin expression by murine myotubes in culture was provided by the staining of differentiated primary muscle cultures from a normal mouse. Antibodies to dystrophin produced a pattern of staining consistent with the localisation of this molecule to the myotube sarcolemma (Fig. 1A). No such staining was observed when these antibodies were used to examine differentiated C2 cultures (Fig. 1B).

A co-culture of C2 and human DMD myoblasts was constructed to examine the possibility that dystrophin expression by C2 cells may be increased by co-fusion with other myoblasts, which are genetically deficient in dystrophin expression but may nevertheless provide factors which have a positive effect on dystrophin gene expression.

Double staining of C2/human DMD myoblast co-cultures with Mandys 1 (anti-rod domain dystrophin) and Hoechst dye #33258, indicated that all hybrid myotubes, containing at least one nucleus from each species, stained positively for dystrophin (Fig. 2A–D). Within the same cultures, non-hybrid myotubes which contained nuclei from only one species did not stain for dystrophin (Fig. 2E–H). The pattern of staining in these hybrid myotubes was generally similar to that of normal human and mouse controls. However, several examples of hybrid myotubes were observed to exhibit discontinuous patches of staining with dystrophin antibodies which, when compared to the Heochst dye stain, often corresponded closely to the location of C2 nuclei.

Western blot analysis of dystrophin expression in the C2/human DMD myoblast co-cultures and appropriate control cultures, was performed (Fig. 3) to supplement the evidence provided by immunofluorescence staining. Western blots of differentiated C2 cultures, when probed with dystrophin antibodies (lane 4), revealed the presence of a faint protein band of 430 kDa, comparable in molecular weight to dystrophin in normal human (lane 1) and mouse (lane 5) primary cultures. This indicates that C2 cells can produce dystrophin, but at extremely low levels in comparison to primary cultures. A second band of 180 kDa was also observed which was present (with lower intensity) in the mouse control, but not in the human control. This may be a proteolytic product of full-length dystrophin, or a protein product of murine myoblasts which has cross-reacting epitopes with dystrophin.

A similar analysis of hybrid cultures (C2 and human DMD myoblasts) also revealed two protein bands of 430 kDa and 180 kDa (Fig. 3, lane 3), but examination of Western blot autoradiographs by scanning densitometry (Table I) indicated that the quantity of protein in the 430 kDa band was almost 10-fold greater in hybrid cultures than in control C2 cultures. The level of dystrophin expression in hybrid cultures, as examined by this method, was comparable to levels of dystrophin expression in human and mouse primary cultures. As ex-

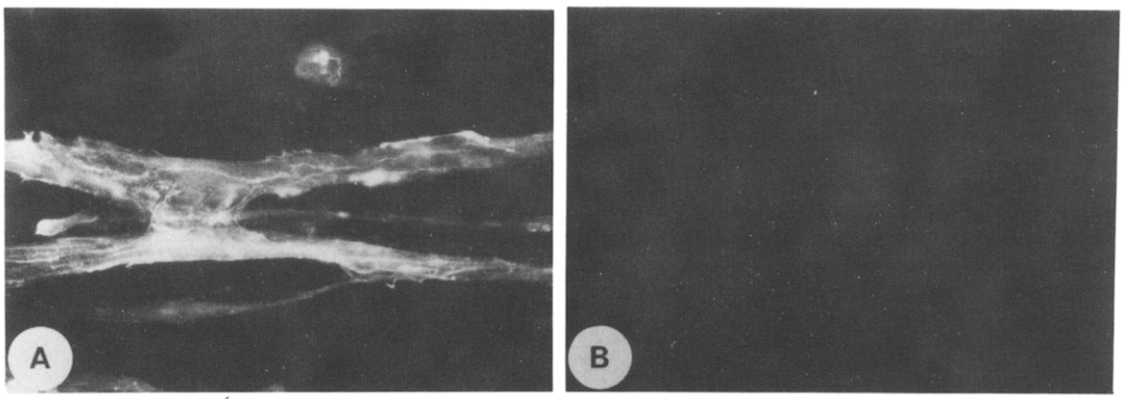

Fig. 1. Immunostains of (A) primary mouse and (B) C2 muscle cultures with Mandys 1 monoclonal antibody to dystrophin, demonstrating a deficiency of dystrophin expression in C2 myotubes. (Magnification × 800)

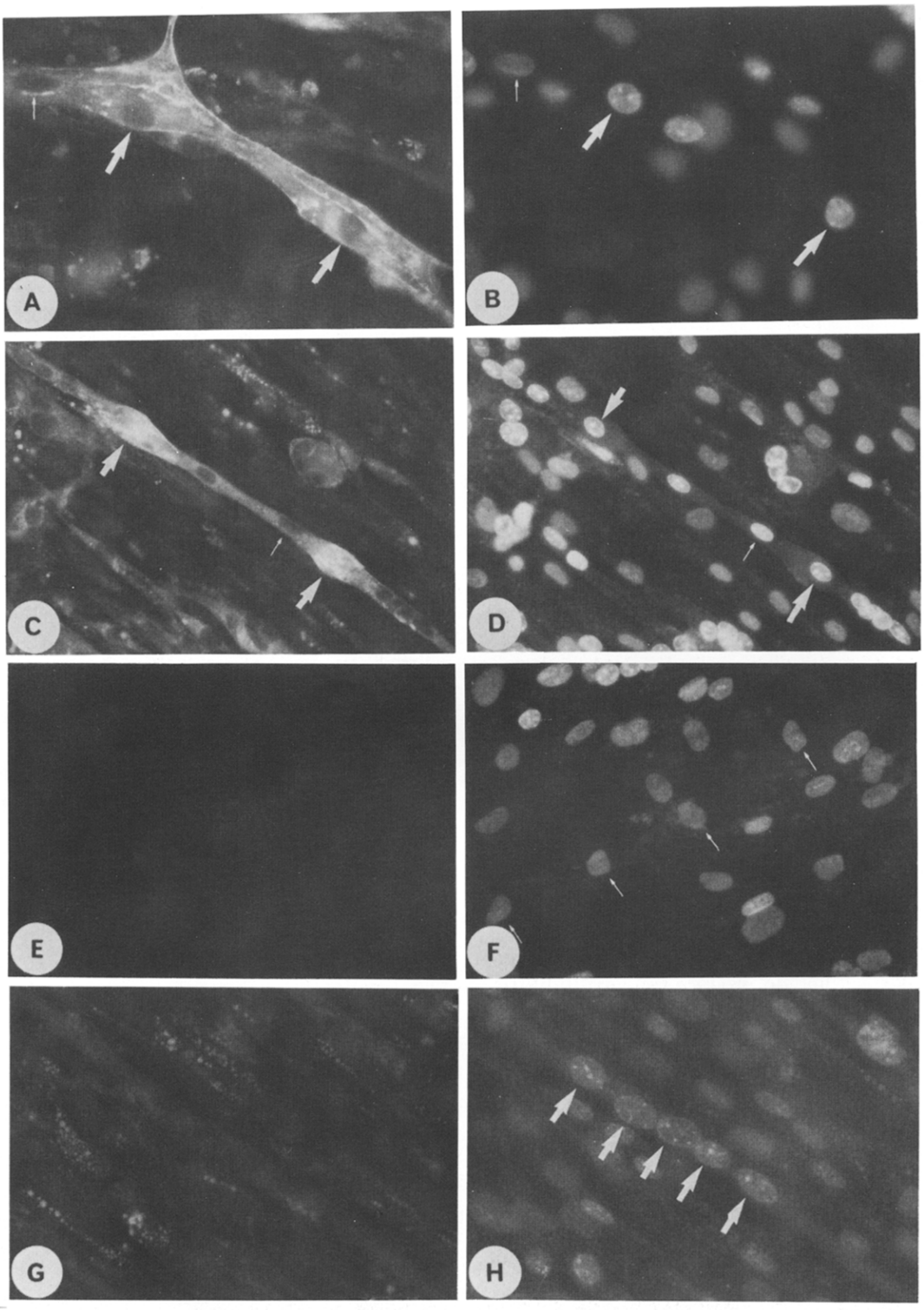

Fig. 2. Dual-immunostaining of differentiated C2/human DMD myoblast co-cultures with Mandys 1 antibody to dystrophin (A,C,E,G) and Hoechst dye #33258 (B,D,F,H). Hoechst dye produces a rough stain with murine nuclei (large arrows) and a smooth stain with human nuclei (small arrows). Hybrid myotubes (A/B, C/D) demonstrate positive membrane staining for dystrophin, whereas no reactivity with Mandys 1 is apparent in non-hybrid myotubes containing either human nuclei only (E/F) or mouse nuclei only (G/H). (Magnification × 800)

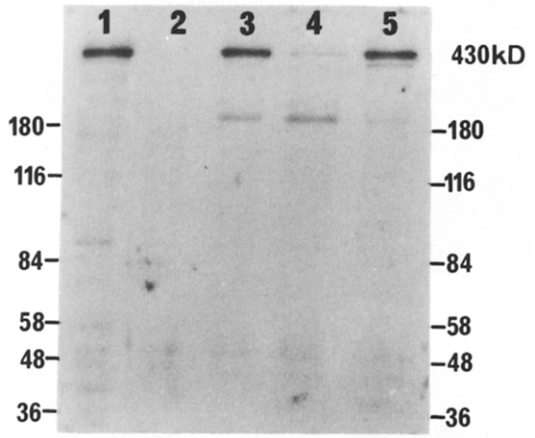

Fig. 3. Western blot analysis of C2/human DMD co-cultures and control cultures with a mono-clonal antibody to dystrophin, 6D3. Lanes 1 and 5 demonstrate levels of full-length dystrophin expression in primary normal human and mouse cultures respectively. Ten-fold lower levels of full-length dystrophin are present in C2 cultures (lane 4) and no dystrophin is apparent in human DMD cultures (lane 2). High levels of dystrophin expression, however, are evident in C2/human DMD co-cultures, comparable to that expressed in primary human and mouse cultures.

pected, no dystrophin was detected by Western blot analysis in control human DMD cultures (Fig. 3, lane 2).

4. DISCUSSION

Klamut et al. [3] demonstrated a deficiency in the production of dystrophin mRNA in C2 cultures compared with that produced by primary myotube cultures. By Western blot analysis and scanning densitometry of autoradiographs we have established that myotube cultures of the C2 cell line do produce full-length dystrophin, but at levels which are 10-fold lower than primary mouse and human cultures. These results suggest that C2 cells do have a functional dystrophin gene, but that they have a deficiency in the positive regulation of dystrophin gene transcription. Transcriptional control is established by the interaction of positive and negative regulatory trans-acting factors with cis-acting promoter and enhancer sequences [17].

Table I

Quantitative analysis of dystrophin expression by scanning densitometry of Western blot autoradiograph shown in Fig. 3

Myotube culture	Relative absorbance of the 430 kDa band
Normal human	10.1
Human DMD	0.0
C2 × Human DMD	9.8
C2	1.0
Normal mouse	10.1

In the present study we show that hybrid myotubes containing C2 and human DMD nuclei express high levels of dystrophin. Since the human DMD nuclei do not have a functional dystrophin gene, this result must represent a dramatic increase in expression of the C2 dystrophin gene. In the same cultures non-hybrid myotubes did not immunostain for dystrophin, clearly indicating that enhanced dystrophin expression by C2 nuclei was occuring as a direct result of the presence of human DMD nuclei within the same myotube. Human DMD nuclei therefore express trans-acting factors which can increase C2 dystrophin expression. These observations indicate that low levels of dystrophin mRNA and protein in C2 cells may result from a deficiency in the expression or activity of an endogenous mouse regulatory factor, which normally has a positive effect on dystrophin gene expression. When the missing factor was complemented by co-fusion with human DMD myoblasts, dystrophin expression by C2 nuclei increased almost 10-fold, to similar levels expressed in normal human and mouse cultures.

The C2 cell line, in conjunction with genomic DNA transfection and expression studies, or by comparative protein binding (gel retardation) studies [18] may therefore be used as a model for the characterisation of positive trans-activating factors of the dystrophin gene. This approach may yield invaluable information about the regulation of dystrophin gene expression, which is currently poorly understood. It may also reveal novel gene regulatory factors with a central role in muscle differentiation. The deficiency of positive gene regulatory factors in C2 cells also demonstrates the possibility that abnormal gene expression may be the result of abnormal gene regulatory mechanisms, rather than a defect of the gene itself. A minority of Duchenne and Becker muscular dystrophy patients for example present with very low expression levels of normal-sized dystrophin, but with no obvious abnormality of the dystrophin gene [19]. Identification of putative gene regulatory factors using the C2 cell line and examination of their expression in normal and dystrophic muscle may provide new evidence for the primary biochemical abnormalities in some muscular dystrophies.

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