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RNA-BASED GENE THERAPIES FOR MYOTONIC DYSTROPHY TYPE 1

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RÉSUMÉ

La dystrophie myotonique de type 1 (DM1) est une maladie neuromusculaire grave qui engendre une perte d'autonomie des patients et diminue leur espérance de vie. Cette maladie est la plus fréquente des dystrophies musculaires chez l'adulte avec une incidence mondiale d'une personne atteinte sur 15 000. Au Québec, cette maladie est d'une importance particulière, car elle touche une personne sur 500 dans les régions du Saguenay et de Charlevoix. La DM1 est causée par l'expansion du triplet CTG situé dans la région 3' non-codante de la myotonine protéine kinase (DMPK). Toutefois, il a été démontré que la grande part des symptômes de la maladie seraient liés à l'accumulation nucléaire de l'ARNm de DMPK portant l'expansion. Ces ARNm mutés se lient à des facteurs nucléaires formant des foci dans les noyaux des cellules DM1, engendrant des effets toxiques sur le métabolisme cellulaire et sur l'épissage alternatif de certains ARNm.

Nos travaux avaient comme but premier d'évaluer si la destruction de l'ARNm mutant de DMPK dans des myoblastes provenant de muscle squelettique DM1 permettrait de rétablir certaines fonctions et caractéristiques normales dans ces cellules. Trois technologies à base d'ARNs: les antisens, les ribozymes et les shRNAs ont diminué avec succès ces niveaux d'ARN mutés. Les ARNs antisens et les ribozymes, contrairement aux shRNA, ont permis un ciblage préférentiel des ARNs mutés de DMPK dans le noyau de myoblastes DM1. Ceci permet donc de maintenir un niveau basal de la protéine DMPK dans les myoblastes, un détail important advenant l'utilisation de ces molécules en thérapie génique chez l'humain. En utilisant les ribozymes, nous avons diminué la quantité et l'intensité des foci ce qui a permis de libérer les facteurs cellulaires se liant aux expansions de CUG. Ceci a eu comme effet de corriger un défaut d'épissage alternatif dans le récepteur à l'insuline. En exprimant de longs antisenses à l'ARNm de DMPK par un oncorétrovirus, nous avons constaté une restauration de la fusion cellulaire, de la capture de glucose ainsi qu'une diminution de CUGBP, un facteur d'épissage alternatif. Nous avons également démontré que la surexpression de hnRNP H, un facteur d'épissage liant les expansions de CUG, permettait

aussi de diminuer les niveaux de CUGBP et ainsi corriger le défaut d'épissage alternatif du récepteur à l'insuline. Ces résultats démontrent donc pour la première fois le lien direct entre la rétention des ARNs mutés, la déplétion nucléaire d'un facteur d'épissage s'y liant et l'exacerbation de certaines caractéristiques du phénotype DM1. La somme de nos observations a permis deux choses importantes: en premier lieu, d'établir un nouveau modèle détaillé expliquant la pathogenèse de la DM1. En second lieu, nos résultats ont permi de valider la pertinence de détruire spécifiquement les transcrits mutés de DMPK afin de développer une thérapie génique efficace pour la DM1.

Marc-André Langlois

Jack Puymirat (Directeur)

John J. Rossi (Co-directeur)

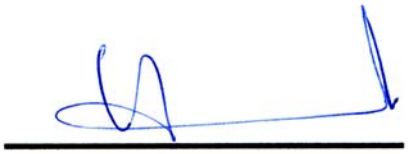
ABSTRACT

Myotonic dystrophy type 1 (DM1) is a severe neuromuscular disease that ultimately causes loss of mobility and premature death. DM1 is the most common muscular dystrophy in adults with a world wide incidence of 1 affected individual in every 15 000. This disease is of special relevance in the Saguenay and Charlevoix regions in Quebec, where 1 in every 500 individuals is a carrier of the mutation. DM1 is caused by the expansion of an unstable CTG trinucleotide repeat located in 3'UTR of the DMPK (DM protein kinase) gene. However, it has been shown that most DM1 symptoms are related to the nuclear retention of mutant DMPK mRNA. These mutant transcripts bind to nuclear proteins and form foci in DM1 cell nuclei. This is though to be the leading cause of metabolical disruptions and defective alternative splicing of several mRNAs observed in DM1 cells.

Our main project objective was to evaluate whether destruction of mutant DMPK mRNA could restore normal phenotype features in DM1 human skeletal myoblasts. The use of three RNA-based approaches: antisense RNAs, ribozymes and shRNAs, all displayed significant reductions in mutant DMPK mRNA. Antisense RNAs and ribozymes, as opposed to shRNAs, allowed specific targeting and destruction of mutant DMPK mRNAs in the nucleus of DM1 myoblasts. This feature thus allows a basal level of DMPK protein expression which is of particular relevance in the advent of developing a gene therapy for DM1. Ribozymes were effective in reducing the number and intensity of foci present in the nucleus of the myoblasts, thus allowing the release of certain CUG-binding proteins. This resulted in restoration of the defective splicing of the insulin receptor mRNA. Antisense RNAs to the DMPK mRNA expressed by an oncoretrovirus restored myoblast fusion, glucose uptake and lowered nuclear levels of CUGBP, an alternative splicing factor. Over expression of hnRNP-H, an alternative splicing factor that we showed could bind to CUG repeats, also reduces expression of CUGBP and restores defective splicing of the insulin receptor. These results reveal for the first time the intricate link between mutant DMPK mRNA nuclear retention, depletion of a CUG-binding protein that is also a splicing factor

and exacerbation of related DM1 features. In conclusion, our work has allowed to better define the mechanisms involved in DM1 pathogenesis and has validated the relevance of developing a gene therapy that specifically targets mutant DMPK mRNAs.

Marc-André Langlois



Jack Puymirat (Directeur)

John J. Rossi (Co-directeur)

AVANT-PROPOS

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Le doctorat met à rude épreuve ses capacités à surmonter divers échelons de problématiques. Peu importe le sujet du projet ou la discipline, cette épreuve nous confronte à une dualité ultime : d'une part à l'avancement de la science, d'une autre part à l'implacable complexité et diversité de la nature humaine. Le doctorat, de par ses exigences, fait surgir bien des émotions, autant provenant de notre for intérieur que du cru de notre entourage. Pour le réussir, il est donc impératif de savoir équilibrer cette dualité. Car si le balancier s'échappe d'un côté comme de l'autre, le tout est voué à un lamentable échec. Ceci est la plus grande leçon que le doctorat ait pu m'enseigner. Maintenant, il s'agit d'accueillir et de mettre à l'œuvre la philosophie qui sera conférée avec le titre du diplôme.

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Ensuite il y a les amis, les collègues et les gens spéciaux qui ont su me tendre une main de soutien aux moments où mon moral n'avait plus de pouls… Je tiens tout d'abord à remercier deux individus sans qui bien des projets ne seraient pas arrivés à terme, Nan Sook Lee et Dong-Ho Kim. Posdocs lorsque j'ai fait mon stage chez le Dr Rossi, j'ai beaucoup appris de vous, merci de m'avoir transmis de vos connaissances et d'avoir partagé votre expérience. Merci à Christelle, David, Séverine, Salim et Nicolas pour qui leur amitié m'est d'une grande valeur et d'un très grand réconfort… J'ai ensuite une reconnaissance spéciale à témoigner à la stagiaire Christelle Boniface pour ses longues heures passées au labo, ainsi que pour avoir fait un travail exemplaire m'ayant permis d'avancer mes projets à un rythme exponentiel. Merci à tout le personnel de soutien, Caroline Haineault, Pierre Chapdelaine, Maurice Dufour, Ronald Maheux, pour de l'aide indispensable et de judicieux conseils. Finalement, un gros merci à toute l'équipe du Dr Jacques Tremblay, pour vos blagues rigolos, pour vos blagues pornos, pour vos discussions constructives, et celles d'un niveau pour endives; pour vos enzymes (oups…), pour vos pétris, pour vos ordis et vos centris, mais avant tout, pour votre amitié et votre gaieté : une chance que vous y étiez….

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Contributions

Chapitre II: Stability of mutant DMPK mRNA in congenital DM1 myoblasts results in increased nuclear accumulation

J'ai effectué la totalité du travail avec la stagiaire Christelle Boniface, à l'exception de la figure 3B qui a été générée par Denis Furling. J'ai également rédigé l'article. Le Dr Puymirat a supervisé l'ensemble du travail.

Chapitre III: Viral vector producing antisense RNA restores myotonic dystrophy myoblast functions

J'ai effectué une partie de la culture cellulaire et j'ai participé aux expériences de capture de glucose. J'ai participé au design expérimental, à la critique et à la révision de l'article.

Chapitre IV: Hammerhead ribozyme-mediated destruction of nuclear foci in myotonic dystrophy myoblasts

J'ai effectué l'ensemble du travail. Le design et le clonage des ribozymes ont été effectués avec les conseils de Nan Sook Lee dans le laboratoire du Dr Rossi. L'analyse des myoblastes exprimant les ribozymes a été effectuée dans le laboratoire du Dr Puymirat. J'ai rédigé l'article. Le Dr Rossi et le Dr Puymirat ont supervisé le travail.

Chapitre V: Short hairpin RNAs mediate cleavage of nuclear-retained mRNAs

J'ai élaboré le concept initial du travail avec Nan Sook Lee. J'ai effectué la totalité du travail avec la stagiaire Christelle Boniface, à l'exception de la figure 2 qui a été générée par Gang Wang (laboratoire du Dr Rossi). J'ai également rédigé l'article. Le Dr Puymirat et le Dr Rossi ont supervisé le travail.

Chapitre VI: An alternative splicing defect in myotonic dystrophy type 1 (DM1) can be rescued by overexpression of hnRNP H

J'ai apporté l'idée originale du projet. L'identification de la protéine a été faite par Dong-Ho Kim dans le laboratoire du Dr Rossi. J'ai fourni l'expertise en culture de cellules DM1 et les conseils concernant la maladie. J'ai généré les figures démontrant le rôle de hnRNP H dans la DM1 et j'ai participé à la rédaction de l'article. Le Dr Puymirat et le Dr Rossi ont supervisé le travail.

Annexe I: Intracellular ribozyme applications

J'ai contribué aux résultats qui ont permis de générer la figure 2 et j'ai apporté l'expertise de la DM1.

ACKNOWLEDGEMENTS

The doctorate is the hard and strenuous challenge of ones capacities to tackle various situations and problems. No matter what the project or the subject matter is, this challenge will confront you with an ultimate duality: on one hand the struggle to advance the science, on the other hand the hard hitting fact of the complexity and diversity of the human nature. The doctorate is a very demanding challenge that will undoubtedly bring out many strong emotions both from us and from the individuals in our work environment. To succeed, it is critical to carefully balance this duality. Because failure would be inevitable if it were to shift to one side or to the other. This is the most important lesson that I have learned from my doctoral experience. I now understand why this degree is called a doctorate in philosophy.

It is with great satisfaction that this thesis finalizes the accomplishments of 43 months of hard work. This degree was a very rewarding journey as much on a personal level than for the new hope it has fuelled in myotonic dystrophy patients. But I did not confront this challenge alone, and numerous are the individuals that have contributed and helped me along the way. It is now time to acknowledge these persons. I would like, first of all, to express my gratitude to my research director, the Dr Jack Puymirat, to have given me his trust and the latitude necessary for me to carry-out several of my ideas (good and bad, crazy or reasonable). I would also like to thank him to have given me the great opportunity, at numerous occasions, to interact with the international scientific community through various meetings and training abroad. I am indebt to you for a robust training, thank you.

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environment (journal clubs, interactions with other students, postdocs and researchers) that I encountered when training in your laboratory, that I have confirmed my desire to pursue my career in fundamental research in academia. Thank you for everything.

Numerous colleagues and friends have also helped made my doctorate rewarding and enjoyable. First I would like to thanks two scientists without whom many of these projects would not have been possible, Nan Sook Lee and Dong-Ho Kim, who were postdocs in Dr Rossi's laboratory. I have learned much from you, thank you for having shared much of your knowledge and experience. Thank you to Christelle, David, Séverine, Salim and Nicolas, I deeply cherish your friendships and the support you have given me these last few years. Next I would like to thank Christelle Boniface, a trainee in the laboratory. Because of your long hours of hard and productive work, you have helped me advance my projects at an exponential rate. Thanks to all the support personnel, Caroline Haineault, Pierre Chapdelaine, Maurice Dufour and Ronal Maheux, for indispensable assistance and advice. Finally, many thanks to Dr Jacques Tremblay's team, my neighbours, for you friendship, your help and the joyful atmosphere you have deployed in the laboratory.

To my parents Mario and Linda and my brother Richard, who have encouraged and supported me, who have made my studies possible. I offer you all my love and gratitude. I would not have succeeded without you. To Françoise, Richard and Suzie, my surrogate family, I wish to thank you for your kindness, generosity and for your support. It has been a pleasure to share these past years with you.

To Catherine… what can I say to you? If there were a Nobel Prize in Psychology-Patience-Support-Diplomacy-and-Indulgence you would undoubtedly be its recipient! Catherine, you have been my source of joy since I have met you, my light in dark places and my daily dose of good humour. Thank you is not sufficient to express my gratitude to have been there for me…. And to still be here for me today. I dedicate this thesis to you…

Contributions

Chapter II: Stability of mutant DMPK mRNA in congenital DM1 myoblasts results in increased nuclear accumulation

I am responsible for the complete work relating to this paper, except for figure 3B that was generated by Denis Furling. The research trainee Christelle Boniface assisted me in the experimental procedures. I am the author of the manuscript. Dr Puymirat supervised and commented the research.

Chapter III: Viral vector producing antisense RNA restores myotonic dystrophy myoblast functions

I did part of the cell culture and contributed to the glucose uptake experiments. I participated in experiment design as well as in the critical reviewing of the manuscript.

Chapter IV: Hammerhead ribozyme-mediated destruction of nuclear foci in myotonic dystrophy myoblasts

I am responsible for generating all the results of this article. Ribozyme design and cloning was done in Dr Rossi's laboratory with the assistance of Nan Sook Lee. Analysis of myoblasts expressing the ribozymes was done in the laboratory of Dr Puymirat. Dr Rossi and Dr Puymirat supervised and commented the work.

Chapter V: Short hairpin RNAs mediate cleavage of nuclear-retained mRNAs

The initial project idea was elaborated in conjunction with Nan Sook Lee. I am responsible for all the results of this report, except for figure 2 that was generated by Gang Wang (Dr Rossi's Laboratory). The research trainee Christelle Boniface assisted me in carrying-out

the experimental procedures. I am the author of the manuscript. Dr Rossi and Dr Puymirat supervised and commented the work.

Chapter VI: An alternative splicing defect in myotonic dystrophy type 1 (DM1) can be rescued by overexpression of hnRNP H

I brought forth the initial project idea. HnRNP-H identification was carried out by Dong-Ho Kim and performed in Dr Rossi's laboratory. I provided expertise in DM1 cell culture and generated figures relating to phenotype restoration in DM1. I participated in writing and commenting the manuscript. Dr Rossi and Dr Puymirat supervised the work.

Appendix I: Intracellular ribozyme applications

My participation in this review consisted in generating figure 2 and in providing some background on myotonic dystrophy.

«Le peu que je sais,
c'est à l'ignorance que je le dois»

- Sacha Guitry

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3'UTR: 3' untranslated region
AAV: Adeno-associated virus
Ad: Adenovirus
ADAR: Adenosine deaminase that act on RNA
ALS: Amyotrophic Lateral Sclerosis
AO: 2'-O-methyl phosphorothioate antisense oligodeoxynucleotide
ApoC2: apolipoprotein C2
AS: Antisense
AS-ODN: Antisense oligodeoxynucleotide
BMP-4: Bone morphogenic protein 4
Bp: Base pairs
CDM: Congenital myotonic dystrophy
CK: Muscle creatine kinase
CKMM: Muscle-specific creatine kinase
Clc-1: Chloride channel-1 protein
CMT: Charcot-Marie-Tooth disease
CNS: Central nervous system
CTL: Cytotoxic T lymphocyte
cTNT: Cardiac troponine T
DM: Myotonic dystrophy
DM1: Myotonic dystrophy type 1
DM2: Myotonic dystrophy type 2
DMD: Duchenne muscular dystrophy
DMPK: Myotonic dystrophy protein kinase or *dystrophia myotonica* proteinase kinase
DNA: Deoxyribonucleic acid
dsRNA-BP:Double strand RNA binding proteins
dsRNA: Double-stranded RNA
eIF2α: Eukaryotic initiation factor 2α
EDMD: Emery-Dreifuss muscular dystrophy
EGS: External guide sequence
FA: Friedreich's ataxia
FISH: Fluorescent *in situ* hybridization
FSH: Follicule stimulating hormone
HIV: Human immunodeficiency virus
hnRNP: heterogeneous nuclear ribonucleoprotein
IFN: Interferon
IGS: Internal guide sequence
ITR: Inverted terminal repeats
IR: Insulin receptor
LGMD: Limb-Girdle muscular dystrophy
LTR: Long terminal repeats
MF-AS: Morpholino antisense
MG: Myasthenia Gravis

miRNA: Micro RNA
miRNP: miRNA-protein complex
MLV: Murine leukemia virus
MSA: Spinal muscular atrophy
MTMR1: Myotubularin-related protein 1
MyHC: Myosin heavy chain
Nt: Nucleotide
ODN: Oligodeoxynucleotide
OligoA: Oligoadenylate
OPMD: Oculopharyngeal muscular dystrophy
RFLP: Restriction Fragment Length Polymorphism
PKR: Protein kinase R
PNA: Peptide nucleic acid
Pol II/III: RNA polymerase II/III
PROMM: Proximal myotonic myopathy
PS: Phosphorothioate
PTGS: Post-transcriptional gene silencing
RBZ: Ribozyme
RISC: RNA-induced silencing complex
RNA: Ribonucleic acid
RNAi: RNA interference
SCA8: Spinocerebellar ataxia type 8
SIN: Self-inactivating virus
shRNA: Small hairpin RNA
siRNA: Small interfering RNA
siRNP: SiRNA-protein complex
snRNA: Small nuclear RNA
snoRNA: Small nucleolar RNA
ssRNA: Single-stranded RNA
stRNA: Small temporal RNA
ZNF9: Zinc finger protein 9

CHAPTER I

Myotonic dystrophy and potential molecular therapeutic approaches

Introduction to muscular dystrophies

Neuromuscular disease is a broad term relating to all disorders affecting both muscle and the spinal cord and/or peripheral nerves. Muscular dystrophies are related to a group of over 30 different and defined myopathies that all share muscle weakness caused by a genetic defect as a common feature. However, these dystrophies may or may not display a neurological involvement. Proximal muscle weakness is the general characteristic shared by most muscular dystrophies which ultimately leads to loss of mobility thus confining the patients to a wheelchair. Myotonic dystrophy is one exception where distal muscle groups are altered but the effects are just as severe. While most forms of muscular dystrophies are progressive with symptoms worsening with time (adult forms), some forms manifest themselves at birth (congenital) with symptoms that are generally much more severe such as developmental and mental retardation. Some diseases like Duchenne muscular dystrophy (DMD) and congenital myotonic dystrophy (CDM) lead to premature death from respiratory or cardiac failure in children and young adults.

The molecular, metabolic or neurological defects responsible for myopathies alter various aspects of muscle structure, metabolism, function and innervation. Table 1 summarizes some of the most frequent muscular dystrophies and the defect responsible for them. While some myopathies occur spontaneously, like in one third of DMD patients for example, most muscular dystrophies have an important hereditary component. Although prenatal genetic testing is now becoming widely available for couples with a familial background of muscular dystrophy, transmission of a recessive form often comes from unsuspecting parents with no prior documented cases of the disease in either family. Because of the complexity of the defects that cause the muscular dystrophies and the large number of muscle groups that are affected, there are no definite cures yet available today.

Table 1: Summary of most frequent muscular dystrophies

Disease	Inheritance	Defect
Becker and Duchenne muscular dystrophy (DMD)	Two thirds of cases are X-linked recessive. One third are caused by new mutations.	Lack of the structural protein dystrophin
Emery-Dreifuss muscular dystrophy (EDMD)	X-linked recessive or dominant	Lack of nuclear protein emerin
Limb-Girdle muscular dystrophy (LGMD)	X-linked recessive	Several mutations can cause LGMD. Lack of sarcoglycans, calveolin, dysferlin, and others…
Myotonic dystrophy (DM)	Autosomal dominant	CTG expansion in the DMPK gene. Toxic RNA gain-of-function
Oculopharyngeal muscular dystrophy (OPMD)	Autosomal dominant	Alteration of the nuclear protein PABP2

Part I: Myotonic dystrophy

1.1. Steinert's myotonic dystrophy

Myotonic disorders are diseases in which symptomatic individuals have difficulty in relaxing a muscle group after contraction. One hallmark feature of these diseases is the difficulty of affected individuals of releasing grip on objects like tools, cups and door knobs for example (Harper, 2001). In 1876, came one of the first documented accounts of a myotonic disorder from Dr Julius Thomsen by studying his most famous patient, himself. The disease he described, known today as *Thomsen's myotonia congenita*, was of hereditary nature, caused myotonia and muscle hypertrophy but was not progressive in nature. Since no muscle weakness is observed in this disease, myotonia congenita is not considered to be a muscular dystrophy. Some thirty years later, in the early 20th century, another myotonic disorder was described with much more pronounced and degenerative symptoms. Myotonic dystrophy or *dystrophia myotonica* was first described by a German physician by the name of Hans Steinert in 1904 (Harper, 2001). He was the first physician to publish a detailed account of a new myotonic disorder in which he noted symptoms such as muscle weakness and wasting, facial weakness, ptosis, selective atrophy of the sternomastoids in the neck and testicular atrophy which underlined an endocrine disturbance. Steinert also conducted pathological studies on an autopsied case where he noted extensive fibrosis and degeneration of skeletal muscle. While Steinert died in 1910 shortly after publication of his paper, others by the names of Batten and Gibb pursued the work and clearly established myotonic dystrophy as a distinct degenerative neurological disorder. They documented that the disease affected several family members and all had the characteristic symptoms of progressive difficulty in walking with weakness of the legs and grip and also a striking lack of expression due to weakness of the facial muscles. Over the past century, through progress in medicine and technology, several clinical and physio-pathological discoveries helped paint a more defined picture of the characteristic symptoms of myotonic dystrophy. It was not however until 1992, that the genetic defect was coined by several teams showing that an unstable CTG repeat expansion in the 3'untranslated

region of a gene could be the molecular basis for the DM pathology (Aslanidis et al., 1992; Brook et al., 1992; Buxton et al., 1992b; Fu et al., 1992; Harley et al., 1992a; Mahadevan et al., 1992).

Myotonic dystrophy is the most frequent muscular dystrophy in adults with a generalized worldwide frequency of one affected individual in every 15 000 (Harper, 2001). Some regions of the world show however elevated frequencies of the disease. Of special relevance to Canadians, the Saguenay region in Quebec shows one of the most elevated frequencies in the world where 1 individual in every 500 is a carrier of the mutation. The population in the Saguenay region shows high frequencies of several genetically inherited disorders mainly caused by a phenomenon called the founder effect. A founder effect relates to the transmission of a genetic mutation by a single individual living in a secluded population to his progeny. The Saguenay region was colonized in the 17th century by a small group of French settlers. It is now known through genealogical studies that most cases of myotonic dystrophy originate from a single common ancestor (Harper, 2001). The Saguenay region is located in Northern Quebec and was generally inaccessible by common means of transportation for most of the winter period, until the avenue of roads and railways in the early 20th century. Because weak immigration to this remote region did not contribute to diversify the genetic pool, the population remained very homogenous and the mutation was widely distributed throughout the population.

What makes myotonic dystrophy such a devastating neuromuscular disease is that it is dominantly inherited and the symptoms occur at earlier ages with every generation, this phenomenon is known as anticipation. Also, the sex of the affected parent has an important role as to the severity of the disease since almost all congenital forms of the disease appear when the mother is the carrier of the mutation. These are some reasons why genetic testing within families known to carry the mutation is crucial.

1.1.1. The symptoms

There are three typical forms of myotonic dystrophy established according to the symptoms and the age of onset. Adult onset myotonic dystrophy is the most common and the less severe form of the disease with symptoms appearing in the early twenties to the late forties. Congenital myotonic dystrophy is the most severe form of the disease with symptoms present at birth. Childhood myotonic dystrophy is when the disease first appear early in life. Although less severe than the congenital cases, this form can be associated with mental retardation and retardation in muscle development. Childhood myotonic dystrophy will not be addressed here.

1.1.1.1. Adult onset myotonic dystrophy

What is striking about myotonic dystrophy is the wide variability in both the symptoms and the age of onset. This neuromuscular disease not only alters normal skeletal muscle functions but also disrupts smooth muscle function, the cardio respiratory system, the endocrine system, the eyes and also the brain and personality (Harper, 2001). Myotonic dystrophy must thus be seen as a generalized disorder and not just one of the skeletal muscles. Table 2 resumes the clinical symptoms that are most frequently encountered within various organs and systems in patients with the adult onset form of myotonic dystrophy. Although many symptoms can be present simultaneously in any given affected individual, the severity of the disease and the extent of the symptoms is often dictated by the age of onset (Harper, 2001; Moxley, 1992). Other than the apparent clinical symptoms are the histological abnormalities in the tissues of myotonic dystrophy patients, especially in the eyes and muscle:

- **Increased central nuclei (centronucleation)**
- **Type 1 fiber atrophy**
- **Ringed fibres**
- **Sarcoplasmic masses**
- **Nuclear chains**

- **Retinal degeneration**
- **Corneal lesions and crystalline lens opacity**
- **Cataracts**

1.1.1.2. Congenital myotonic dystrophy

The severe form of the disease that is present at birth is called congenital myotonic dystrophy. This form is almost exclusively seen when the mother is the carrier of the disease (Harper, 2001). This devastating form of myotonic dystrophy is often regarded as a distinct neuromuscular disease altogether because of the likely involvement of developmental disturbances, the extreme severity of the symptoms and the heavy neurological component that is not present in adult onset cases. This form is associated with hypotonia and also cardiac and respiratory complications that very often lead to the death of the neonate (Harper, 2001). Neurological features include mental retardation, delayed motor development and difficulties in feeding, swallowing and speech. The condition of the few neonates that survive into childhood seems to improve at first, but rapidly regresses once the adult and degenerative features of the disease appear. Individuals with the congenital form are heavily disabled throughout their life and do not live beyond 30 years of age (Harper, 2001).

Table 2: Myotonic dystrophy clinical symptoms in adults

System / Organ	Defect
Endocrine	• Insulin resistance • Testicular atrophy • Increased FSH levels
Eyes	• Cataracts • Ptosis • Retinal degeneration
Heart	• Conduction defects • Cardiac arrhythmias
Respiratory	• Weakness and myotonia of the diaphragm and respiratory muscles • Abnormal pharyngeal and oesophageal contractions • Bronchial aspiration • Alveolar hypoventilation
Neurological / Brain	• Mental retardation in congenital forms • Hypersomnia • Variable cognitive impairment • Personality : increased apathy, avoidance and passive-aggressive behaviour (Delaporte, 1998)
Skin	• Premature balding
Smooth muscle	• Widespread involvement in gastrointestinal tract • Delayed gastric emptying • Sphincter laxity • Incoordinate contractions of the uterus during labour
Skeletal muscle	• Myotonia • Muscle weakness and wasting • Distal weakness of the limbs • Weakness of facial, jaw, tongue and neck muscles • Severe weakness and wasting of sternomastoids

1.1.2. The *dystrophia myotonica* mutation

The first attempts of positioning the DM mutation were carried out in the early 70's when linkage was first established with the Lutheran blood group utilizing family studies in the USA and Britain (Harper et al., 1972). The presence of the Lutheran antigen a+ Lu(a+) was consistently passed down with the DM mutation in the studied families (Harper et al., 1972). In the following years, several other loci (ABH secretor locus, peptidase D, Lewis blood group, complement C3, ApoE) were all linked with transmission of the DM mutation (Harper et al., 1972; O'Brien et al., 1983; Schrott and Omenn, 1975; Sistonen, 1984; Whitehead et al., 1982). Assignment of the complement C3 loci to chromosome 19 in 1982, made it clear that DM mutation was also located on that chromosome (Whitehead et al., 1982). The DM mutation was then further narrowed down to the 19p13-19q13 region when linkage to its closest markers, the genes for apolipoprotein C2 (ApoC2) and the muscle-specific creatine kinase (CKMM) were established by studying French Canadian, British French and Dutch families (Brunner et al., 1989; Shaw et al., 1985). The final positioning of the DM gene was done in 1991 by Harley and co-workers by establishing that the DM gene lies in region 19q13.2-q13.3, telomeric to the ApoC2 and CKMM genes (Fig. 1) (Harley et al., 1991).

From then, several teams put much effort in isolating and constructing DNA libraries in lambda phages, yeast artificial chromosomes and cosmids (Aslanidis et al., 1992; Buxton et al., 1992a; Harley et al., 1992b). By Restriction Fragment Length Polymorphism (RFLP) studies, a genomic segment was identified that showed length variations in myotonic dystrophy patients (Aslanidis et al., 1992; Buxton et al., 1992b; Harley et al., 1992a). It was in early 1992, through positional cloning techniques, that the mutation responsible for myotonic dystrophy was first identified as an instable CTG expansion (Aslanidis et al., 1992; Brook et al., 1992; Buxton et al., 1992b; Fu et al., 1992; Harley et al., 1992a; Mahadevan et al., 1992). A very interesting observation came from Mahadevan and co-workers when they showed that the CTG expansion was located in the 3' untranslated region (3'UTR) of dystrophia myotonica protein kinase gene (DMPK) in 98% of patients

clinically diagnosed with myotonic dystrophy (Mahadevan et al., 1992). Although the function of DMPK is still to date unclear, it was challenging at the time to comprehend how this expansion both on the DNA and transcribed to the RNA was responsible for the cellular and molecular defects seen in dystrophic cells.

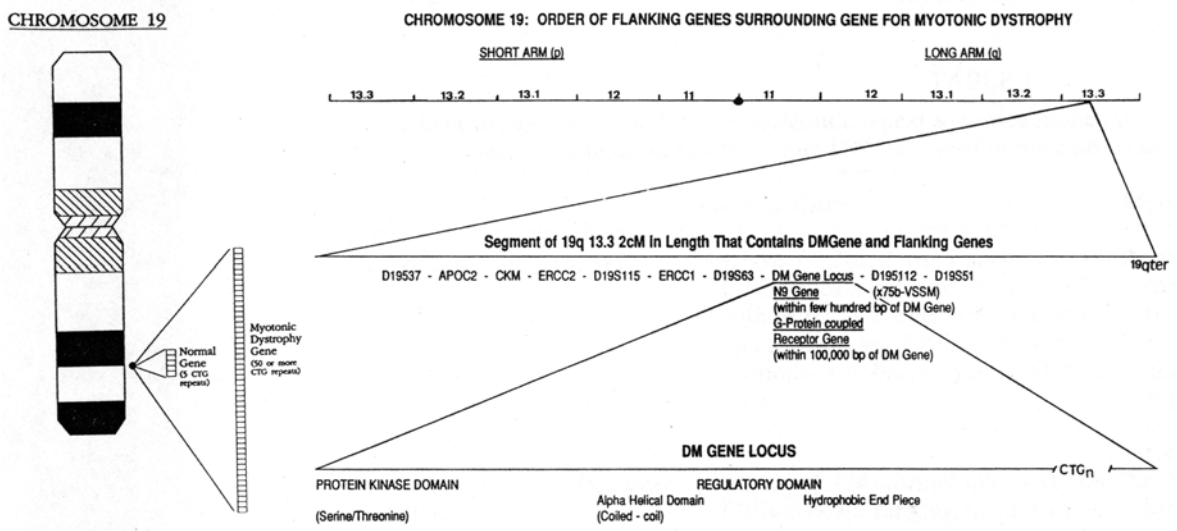

Figure 1. The DM1 gene locus.

The left panel shows a schematic representation of chromosome 19 and the approximate location of the DM1 gene locus with or without the CTG expansion. The right panel shows flanking genes and probes used to locate the DM1 gene locus. (Illustration from.(Moxley, 1992)).

The discovery of the mutation causing myotonic dystrophy came at a turning point in genetics when a number of diseases affecting the central nervous system (CNS) were being identified as trinucleotide repeat-related disorders. The previous year, in 1991, the CGG trinucleotide expansion causing fragile X syndrome had been identified (Verkerk et al., 1991) and the following year, in 1993, the CAG repeat expansion responsible for the toxic polyglutamine tract causing Huntington's disease was also identified (Group, 1993).

Several trinucleotide diseases identified to date share several key features, reviewed in (Brice, 1998; Martin, 1999; Paulson and Fischbeck, 1996):

1) **Inheritance is autosomal dominant or x-linked (except for Friedrich's ataxia, which is autosomal recessive)**

2) **Severity of the disease is variable but generally correlates with increased expansion length**

3) **Trinucleotide repeats are unstable when they reach a certain threshold and generally undergo expansion when transmitted, a premise to anticipation**

4) **Expanded repeats are polymorphic when inherited, but are transmitted stably when under the critical threshold**

Trinucleotide diseases can be further broken-down within 2 categories, diseases where the repeats are translated into protein or not (Table 3). When the repeats are not translated, the diseases are characterized by much larger expansions and usually multiple systems are affected. It is fascinating to see how a similar type of mutation can generate on one hand common clinical symptoms which is CNS degeneration, especially for the group of translated expansion disorders coding for a polyglutamine tract and on the other hand disease-specific alterations. Both myotonic dystrophy and SCA8, for example, are caused by a transcribed but untranslated CTG repeat. Although SCA8 patients have generally much fewer CTG repeats than in myotonic dystrophy, very few clinical symptoms are shared by both diseases.

Table 3: Trinucleotide repeat diseases

Disease	Repeat	Translated	Threshold
• Fragile X types A-E	CGG	No	Normal: 6-52 Pre-mutation: 60-200 Disease: 200-2000
• Myotonic Dystrophy Type I	CTG	No	Normal: 3-49 Disease: 50-3000
• Spinocerebellar ataxia type 8 &12 (SCA8 & 12)	CTG	No	Normal: 16-37 Disease: 90-152
• Friedreich's ataxia (spinal bulbar muscular atrophy)	GAA	No	Normal: 7-22 Disease: 100-2000
• Huntington's disease	CAG	Yes	Normal: 11-34 Disease: 36-121
• Kennedy's disease	CAG	Yes	Normal: 11-42 Disease: 40-62
• Spinocerebellar ataxia types (1-3,6, 7)	CAG	Yes	Normal: 7-36* Disease: 37-130*
• Dentatorubral-pallidoluysian atrophy	CAG	Yes	Normal: 7-25 Disease: 49-88

**General approximation. Each SCA disease has its own defined clinical threshold (Martin, 1999)*

1.1.3. Anticipation and somatic instability

Anticipation in myotonic dystrophy and also in other triplet repeat diseases is a hallmark clinical feature that has been documented for more than 90 years (Harper, 2001; Moxley, 1992). It was not until the early 90's, once the mutation had been identified, that extensive studies were conducted to monitor several key components of anticipation in myotonic dystrophy. These studies were done on several families and looked at parameters such as the age at onset of the affected parent, the age at onset of offspring, the number of repeats in affected parents and offspring and the influence of the sex of the affected parent on the outcome of the disease in the offspring (Ashizawa et al., 1994; Ashizawa et al., 1992a;

Ashizawa et al., 1992b; Harley et al., 1993). The results revealed that although contraction of the mutation was observed in 10% of cases when the father carried the mutation and in 3% of cases when the mother had the mutation, the general tendency was that offspring had an increase in the length of the repeat tract and severity of the disease (Ashizawa et al., 1992b). The increment in CTG repeats in children with myotonic dystrophy is closely related to the length of the repeats in the parents, especially if the mother is a carrier of the mutation (Barcelo et al., 1993; Harper, 2001). Observations on families with myotonic dystrophy allowed thresholds linking CTG repeat number amplification and disease severity to be established (table 4).

Table 4: Critical CTG expansion thresholds for myotonic dystrophy onset and symptoms

Number of CTG repeats	Phenotype	Comments
3 to 49	Normal individuals	Over 90% of the general population has less than 35 CTG repeats in the DM1 locus.
50 to 80	Very mild or no apparent symptoms except cataracts	Expansions in this range are incremental from generation to generation.
80 -1000	Full mutation, adult onset	80 CTG repeats is the threshold for saltatory amplification.
600 and more	Full mutation, adult onset	High risk of transmitting the congenital form if the mother is the carrier.
1000 to 3000	Generally congenital	Such expansions are most frequently seen in congenital cases.

In order to better understand how the severity of myotonic dystrophy increases within the lifespan of an affected individual, and why the children with myotonic dystrophy have generally longer repeat lengths than their parents, it is necessary to look at how the CTG repeat tract is replicated. To shed light on the molecular mechanisms of DNA replication, several teams have studied how these CTG repeats behave in *Escherichia coli* (Jaworski et al., 1995; Kang et al., 1995; Samadashwily et al., 1997; Sarkar et al., 1998). Their first observation was that both expansions and deletions exist in *E.coli* depending on which strand lies the expansion (Iyer and Wells, 1999; Kang et al., 1995). A CTG repeat tract tends to expand when located on the leading template rather than on the lagging strand (Iyer and Wells, 1999; Kang et al., 1995). Figure 2 panels A and B displays a model of current beliefs on how this may occur. Although this model gave, in 1995, the first insight on how CTG repeats could expand from generation to generation, it could not explain how there is sometimes a dramatic amplification when the mutation is transmitted from mother to child, as in congenital cases for example.

In 1998, a paper by Sarkar and colleagues brought new insight to somatic instability of CTG repeat tracts. This work brought to light mechanisms that may be implicated in the bimodal pattern of CTG amplification (Sarkar et al., 1998) where small CTG repeats ($n < 80$) show incremental augmentation when inherited as compared to the large amplifications seen when the parent has relatively large expansion (>200). The main conclusions of this work proposed that expansions smaller than a single Okazaki fragment in humans (< 100 – 200 bp) is susceptible to an incremental amplification but the much larger amplifications were caused when several CTG-encoding Okazaki fragments were necessary to complete the strand replication (Sarkar et al., 1998). These Okazaki fragments could undergo slippage from being unanchored at both ends by a non-repetitive sequence. The combined effect of having several Okazaki fragments containing long CTG hairpin expansions that could evade normal DNA repair during mitosis is the standing model for these large expansion amplifications seen when affected individuals reach the critical threshold of approximately 80 CTG repeats (240 bp). CTG amplification in this *E.coli* model necessitated however loss of SbcC, a protein that modulates cleavage of single-stranded

DNA and degradation of duplex DNA from double-strand breaks. It is still not known today if disruption of a homologous protein to SbcC in humans or if the misregulation of other elements of the mismatch repair system like Msh3 and Msh6 are also implicated at some level for CTG amplification in myotonic dystrophy (Sarkar et al., 1998; van den Broek et al., 2002).

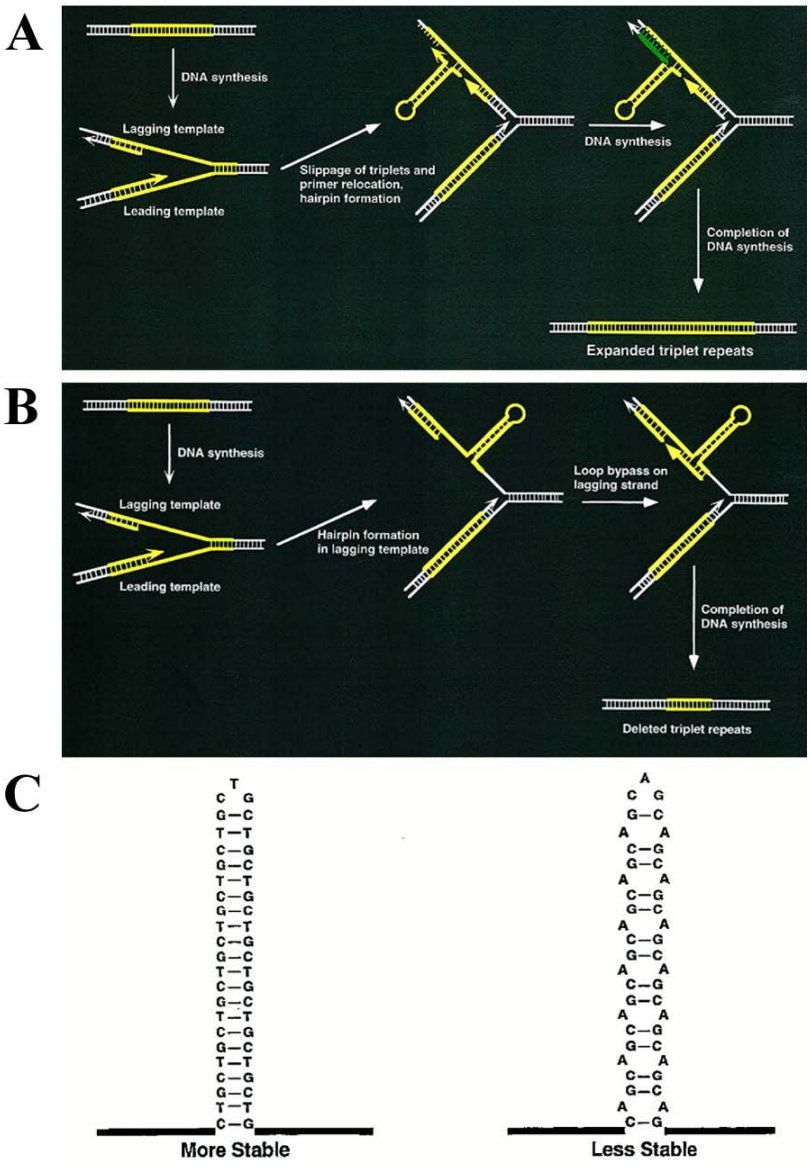

Figure 2. Model for CTG expansion and contraction mechanisms.

(A) *A possible mechanism for expansion is that primer relocation and polymerase slippage when transcribing CAG repeats induces the formation of a CTG hairpin loop on newly*

synthesised DNA. The size of these loops will dictate the length of the expansion in the replicated strand. ***(B)*** *A model for deletions (or contractions) where the replication fork skips a strong CTG hairpin secondary structure present in the template DNA. This skipping will result in the loss or reduction of the CTG tract in the synthesized DNA.* ***(C)*** *The predicted base-paring structure for CTG and CAG repeats. CTG repeat hairpin structures are far more stable because of the weak repulsion of T-T pairs compared to A-A pairs (Smith et al., 1995). (Illustration from (Wells, 1996))*

Because of the unstable nature of the repeat tract, it is expected that dividing cells are subject to increasing expansions with every division. This phenomenon of somatic instability underlines the very basis of anticipation in the disease and may explain why the severity of myotonic dystrophy increases in time. Patient studies focussed on somatic instability assessed two important points: variations in the mutation length between different tissues and the variation of the mutation length in a single tissue at different points in time.

Five important elements derived from patient studies highlight the unstable nature of the CTG repeats (Martorell et al., 1997):

1) **The expanded allele appears as a smear on Southern blots**
2) **Identical twins affected by myotonic dystrophy have different expansion patterns**
3) **Immortalized lymphocytes from patients show increased expansions in culture**
4) **Repeat lengths in patients blood and tissue increase with time**
5) **Repeat lengths in DNA vary from tissue to tissue in a same patient**

These important elements paint out myotonic dystrophy as a disease where repeat sizes increase at different rates in different tissues. Hence, this dynamic process may explain the

age-dependent and tissue dependent phenotypic manifestations of the disease (Khajavi et al., 2001).

Somatic instability in myotonic dystrophy seems to occur in a time-dependent fashion. Studies conducted on congenitally affected fetuses and neonates revealed that repeat heterogeneity between organs and tissues appears after 16 weeks of development (Martorell et al., 1997). This timing coincides with the second trimester of development and the second wave of myogenic development (Martorell et al., 1997). Following the 16th week of development, expansion heterogeneity sets-in and continues to expand throughout the life of the affected individual. Progression of the CTG tract instability does not seem to coincide solely with the mitotic activity of the tissue since mean expansion length in lymphocytes and sperm are generally much lower that those seen in muscle, heart or kidney (Anvret et al., 1993; Martorell et al., 1997; Martorell et al., 1998; Monckton et al., 1995; Zatz et al., 1995). It is of interest to note that although the mean repeat length in sperm is lower than that of other tissues, there is extensive variability in repeat length in the male germline including both contractions and even reversions to normal length (Monckton et al., 1995). This variability may be responsible for some observed cases of contractions seen when the father is the carrier of the disease and may also add an alternative explanation to the intergenerational amplification seen between affected fathers and their offspring (Harper, 2001; Monckton et al., 1995).

1.1.4. Molecular alterations and cellular defects

Although the clinical and physiological perturbations of myotonic dystrophy have long been described, the more subtle molecular and cellular defects are much more elusive. It is still not clear today, twelve years after the initial identification of the mutation responsible for myotonic dystrophy, which are the molecular mechanisms involved in causing the vast range of symptoms seen in the disease.

1.1.4.1. The DMPK mRNA

Since the very beginning, it was shown that mutant DMPK RNA levels were altered in the disease. However, conflicting reports brought only confusion to whether mutant DMPK mRNA was reduced, elevated or unchanged in myotonic dystrophy (Carango et al., 1993; Fu et al., 1993; Sabouri et al., 1993; Wang et al., 1995). One group even reported complete absence of mutant DMPK mRNA from congenitally affected fetuses and infants (Hofmann-Radvanyi et al., 1993). Although the reasons for these variations were not understood at the time, it seemed to be clear that the lack or abundance of mutant DMPK mRNA was not the cause but a consequence of the disease.

1.1.4.2. Mutant mRNA retention

Clues to understanding such variability in results came shortly afterwards with elegant work done by Taneja and co-workers showing that mutant DMPK mRNA with large CUG expansions were retained in the nucleus of myotonic dystrophy cells and form discrete foci when revealed by in situ hybridization (Fig. 3) (Taneja et al., 1995). Work performed by Davis and co-workers determined that mutant RNAs are linked to the nuclear matrix and were not exported to the cytoplasm (Davis et al., 1997). They showed by Northern analysis that this retention was not due to disruption of splicing or polyadenylation of the mutant transcripts (Davis et al., 1997). They also proposed that conflicting reports concerning mutant DMPK expression levels were most likely caused by improper RNA extraction and unreliable diagnosis methods.

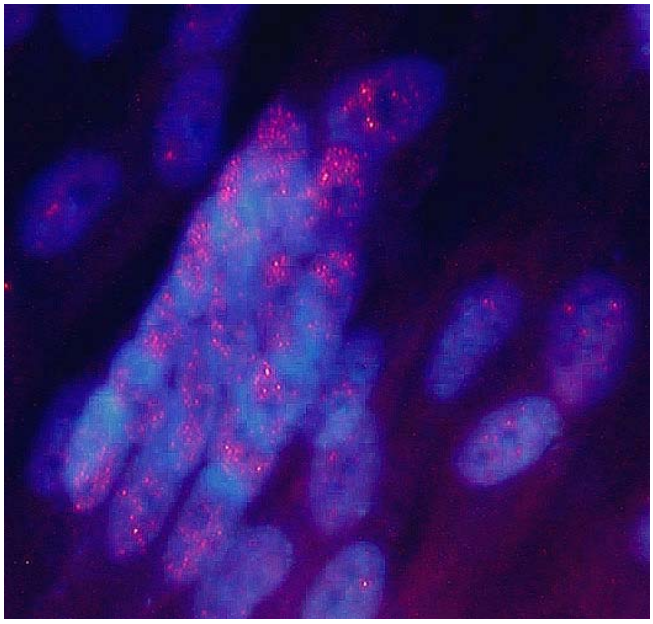

Figure 3. Mutant DMPK mRNA form foci in the nucleus of DM1 myoblasts.

In situ hybridization performed on DM1 myoblasts with 3000 CTG repeats using a Cy3-labeled peptide nucleic acid $(CAG)_5$ probe and DAPI staining. (Langlois *et al*, unpublished data).

1.1.4.3. The DMPK protein

Retention of the mutant DMPK mRNA causes a haplo-insufficiency of DMPK protein since only the normal transcripts are translated. Accordingly, it was demonstrated using specific anti-DMPK antibodies that DMPK protein levels were reduced to 50-57% of the levels in normal myoblasts (Furling et al., 2001a; Furling et al., 2001b). DMPK is a cyclic AMP-dependent serine/threonine protein kinase belonging to the Rho family. Fifteen exons predicts a 70.6 kDa protein with over 7 different tissue-specific splicing isoforms (Fig. 4a) (Groenen et al., 2000; Tiscornia and Mahadevan, 2000; Wansink et al., 2003). However, through strong post-translational modifications, certain isoforms of the protein can achieve an apparent molecular mass of up to ~ 80-86 kDa in skeletal muscle (Bush et al., 2000; Lam et al., 2000; Mahadevan et al., 1993; Wansink et al., 2003). These numerous DMPK

isoforms exhibit cell-type and location-dependent substrate specificities possibly conferring to DMPK different physiological roles (Wansink et al., 2003). Figure 4b depicts the DMPK protein with its various structural domains. The function of these domains still needs to be defined, but one study has suggested that DMPK must be cleaved from it's membrane associated domain in order to adopt an active conformation in the cytoplasm (Bush et al., 2000). Another study from the same group, suggests that phospholemman, a membrane-bound substrate for protein kinase A and C involved in ion transport, is also a substrate for DMPK phosporylation (Mounsey et al., 2000a). Modification of muscle Ca^{2+} and Na^{+} ion channels homeostasis could lead to an alteration of muscle excitability, as seen in myotonic dystrophy (Benders et al., 1997; Mounsey et al., 2000b). At this time, there are but speculations of the true biochemical role of DMPK in healthy individuals or in myotonic dystrophy (Wansink et al., 2003).

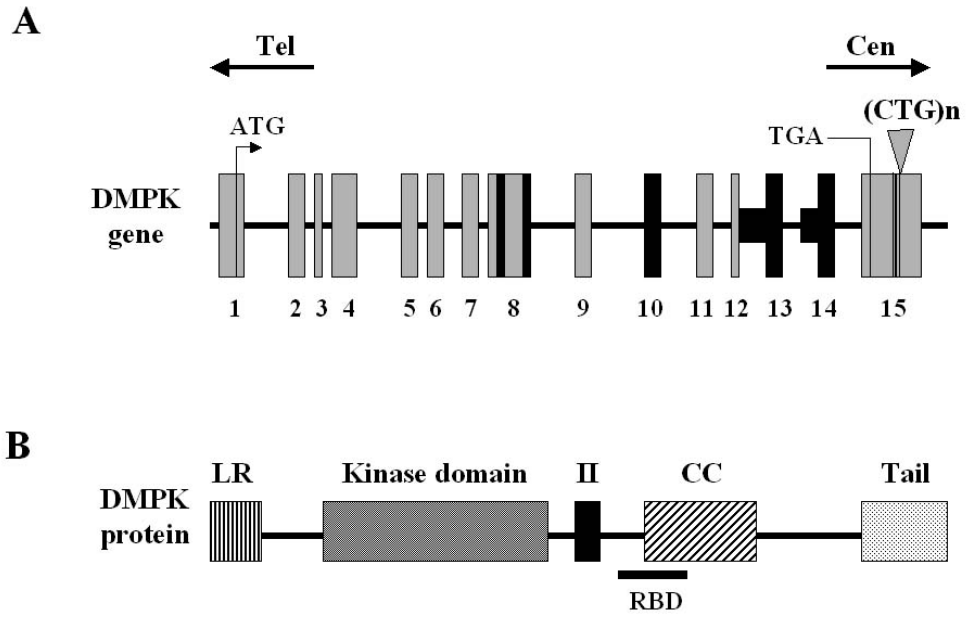

Figure 4 The DMPK gene and protein.

(A) *The DMPK gene consists of 15 exons. The CTG expansion is located in exon 15. Exons are depicted as grey boxes, black boxes represent alternatively spliced exons and small*

white boxes represent cryptic intron segments. ***(B)*** *The DMPK protein is comprised of a N-terminal leucine rich region (**LR**) followed by the serine/threonine kinase domain. Region **II** encodes a VSGGG motif responsible for substrate specificity, followed by a possible Rho-binding domain (**RBD**), a coiled-coil domain (**CC**) and a subcellular localization tail domain (**Tail)** allowing targeting of the protein to mitochondria, endoplasmic reticulum or to the cytosol (Wansink et al., 2003)***.***

1.1.4.4. Animal models

The development of specialized tools like mouse models and cell lines were necessary in order to examine the effects of the myotonic dystrophy mutation in live cells and tissues. In 1996, appeared the first mouse models to help understand myotonic dystrophy (Jansen et al., 1996). In these models, Jansen and colleagues disrupted the endogenous murine DMPK gene and/or overexpressed a normal human DMPK transgene. Their goal was to determine the influence of DMPK misregulation in the disease. Interestingly, the nullizygous mice they generated showed only mild atrophy in head and neck muscles, while the mice overexpressing human DMPK showed copy number-dependent cardiomyopathy (Jansen et al., 1996). In another study, the authors showed that these knockout mice showed cardiac conduction defects (Berul et al., 1999), which is in accordance with the previously published results and decreased muscular strength implicating a role for DMPK in maintenance of muscle structure (Reddy et al., 1996). However, none of these mouse models have exhibited the most prominent features of myotonic dystrophy such as the myotonia, cataracts and severe muscle wasting. These results clearly point out that the DMPK protein itself has but a subtle role in myotonic dystrophy pathogenesis in mice. Since murine and human DMPK share much similarity, it is not expected that DMPK plays any critical role in humans. Also, patients homozygous for the DM mutation do not exhibit symptoms more severe than heterozygous patients with a similar expansion length (Harper, 2001). This implies that the complete lack of DMPK protein in humans does not account for any visible phenotype at first glance.

Two other mouse models were later generated to look at CTG tract stability. The first group used a fragment of DMPK 3'UTR containing 162 CTG repeats (Monckton et al., 1997), the other inserted the whole DM locus (45 kb) containing 55 CTG repeats and adjacent SIX5 and DMWD genes (Gourdon et al., 1997). Both groups arrived at similar conclusions as they observed modest, but significant meiotic and mitotic CTG repeat instability. The best myotonic dystrophy mouse model to date came later when Gourdon's group inserted a 300 CTG repeat expansion in the 45 kb genomic fragment instead of the 55 CTG repeat sequence. (Seznec et al., 2000). These mice show a very similar pathological phenotype as seen in humans:

- **A high level of instability increasing with age in tissues and sperm**
- **Histological muscle abnormalities (atrophy of slow muscle fibers, centronucleated muscle fibers, fibrosis and heterogeneity in fiber diameters)**
- **Myotonia**
- **Formation of foci revealed by a DMPK riboprobe**

A major step in understanding the true nature of myotonic dystrophy came with the development of a mouse model carrying a large CTG repeat expansion (~250) in 3'UTR of the human skeletal actin gene (Mankodi et al., 2000). The aim of that study was to determine if the pathogenic effect of the mutation comes from the RNA or the DNA. What they observed was that mice carrying the expanded repeat developed myotonia, several histological features of myotonic dystrophy (ring fibers, sarcoplasmic masses, centronucleation) and CUG-related foci formation in muscle cells. However, no muscle weakness was reported in these mice. The fact that these mice exhibited the cardinal features of human myotonic dystrophy strongly supported a toxic gain-of-function role for RNA containing the large CUG repeats.

1.1.4.5. Gain-of-function mutation and splicing aberrations

The initial idea of the gain-of-function effect of the RNA in myotonic dystrophy came in 1996 from Timchenko and co-workers when they identified CUG-BP, a heterogeneous nuclear ribonucleoprotein (hnRNP), that could bind to single-stranded CUG repeats and also accumulates in the nucleus of cells from myotonic dystrophy patients (Roberts et al., 1997; Timchenko et al., 1996a; Timchenko et al., 1996b; Timchenko et al., 2001a). CUG-BP is a phosphorylation substrate for the DMPK protein and exists as two phosphorylation isoforms that are ubiquitously expressed in both the cytoplasm and the nucleus (Roberts et al., 1997; Timchenko et al., 1996b). It is the hypophosphorylated isoform that accumulates in the nucleus of cells form myotonic dystrophy patients. The role hnRNPs, such as CUG-BP, is to perform multiple posttranscriptional regulatory functions such as splicing. CUG-BP is a member of the CELF family of RNA-processing factors that regulate alternative splicing (Ladd et al., 2001). Aberrant accumulation of the hypophosphorylated form of CUG-BP in the nucleus has been associated with abnormal splicing of several of its substrate mRNAs, ultimately lending support for a trans-dominant effect of expanded CUG repeats on RNA processing in myotonic dystrophy type 1 (Savkur et al., 2001). However, this accumulation is unlikely to be a titration effect from binding to the CUG repeat tract itself since accumulation of CUG-BP in the nucleus was not shown to be proportional to it's length (Michalowski et al., 1999).

Five pre-mRNAs have been identified to be aberrantly spliced in myotonic dystrophy type 1 tissues and mouse models: tau (Sergeant et al., 2001), myotubularin-related protein 1 (MTMR1) (Buj-Bello et al., 2002), the insulin receptor (IR) (Savkur et al., 2001), cardiac troponine T (cTNT) (Philips et al., 1998) and that of chloride channel 1 (Clc-1) (Charlet et al., 2002; Mankodi et al., 2002). While the role of MTMR1 is still elusive in myotonic dystrophy, disruption of appropriate splicing of cTNT, IR and CLC-1 mRNAs can be associated with symptoms of the disease. It has not yet been demonstrated that the splicing defect of tau is associated with particular symptoms of myotonic dystrophy. However, tau is a microtubule-associated protein implicated in axon and neurite development in the brain (Shahani and Brandt, 2002). Alterations in the balance of tau isoforms may well be

responsible for the abnormal personality traits of myotonic dystrophy patients and could explain the severe involvement of the CNS in congenital cases.

Human cTNT exists as two splicing isoforms with either inclusion or exclusion of exon 5. The isoform that includes exon 5 is normally only expressed during embryonic development in the heart and skeletal muscle, while in adults exon 5 is excluded (Cooper and Ordahl, 1984). Homozygous and heterozygous myotonic dystrophy patients show alternate splicing patterns with a significant increase in cTNT mRNAs with exon 5 inclusion (Philips et al., 1998).

The insulin receptor is composed of two extracellular α and two intracellular β subunits. Alternative splicing of exon 11 generates two isoforms of the α subunit: isoform A (IRA) lacking exon 11 and isoform B (IRB) that includes exon 11 (Fig. 5a) (Seino and Bell, 1989). Expression of the two isoforms is tissue specific depending on glucose requirement of each particular tissue since both isoforms do not have the same signalling capabilities (Kellerer et al., 1992; Moller et al., 1989). The B isoform, that is expressed dominantly in skeletal muscle, adipose tissue and the liver, has been reported to signal more efficiently in response to insulin binding despite having a 2-fold lower affinity for insulin (McClain, 1991; Moller et al., 1989; Mosthaf et al., 1991; Yamaguchi et al., 1993). In myotonic dystrophy, the IR is aberrantly spliced thus promoting the IRA isoform is skeletal muscle (Savkur et al., 2001).

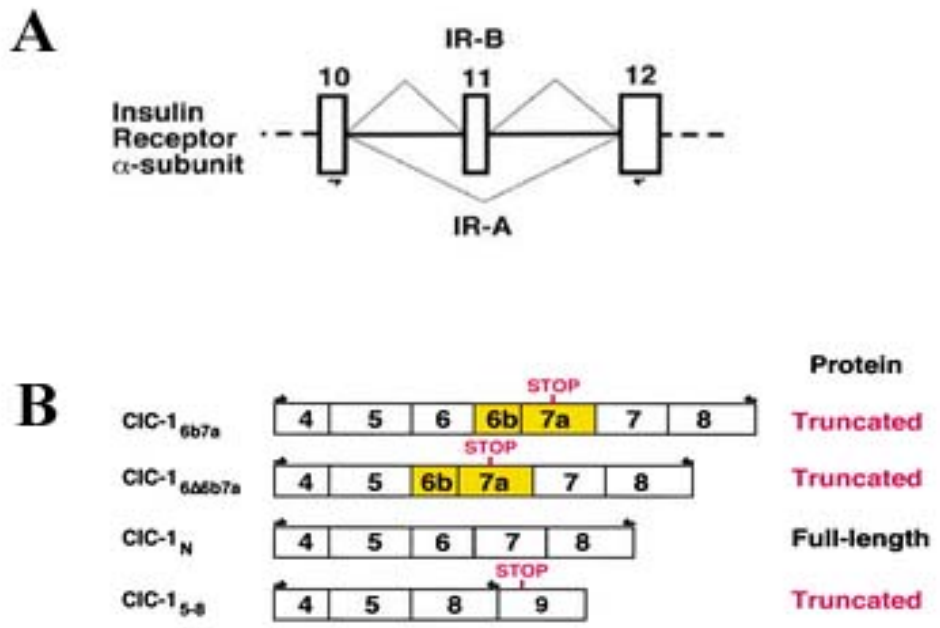

Figure 5 Diagram of the alternative splicing of IR and Clc-1

(A) *Insulin receptor (IR) α-subunit alternative splicing. IR-A is produced with inclusion of exon 11, while the IR-B isoform is produced by exclusion of exon 11. IR-A is the high-signalling isoform normally found in insulin-responsive tissue like skeletal muscle and the liver.* ***(B)*** *Alternative splicing of the chloride channel-1 (Clc-1) mRNA. Aberrant splicing generates 3 isoforms coding for inactive proteins with premature stop codons. (Illustration reproduced from:* ***(A)*** *(Savkur et al., 2001)* ***(B)*** *(Charlet et al., 2002))*

Myotonia is one of the major cardinal features of myotonic dystrophy. The inability to relax muscles after contractions is due to hyperpolarisation of the muscle fibers after ion entry (Lehmann-Horn and Jurkat-Rott, 1999). Several channelopathies such as mutations in the muscle-specific sodium (*SCN4A*) and chloride (*CLCN1*) channel genes are known to cause myotonia in humans and animals (Lehmann-Horn and Jurkat-Rott, 1999). The Clc-1 channel is the product of the *CLCN1* gene and is the major skeletal muscle chloride channel in humans (Lehmann-Horn and Jurkat-Rott, 1999). Since defects in both sodium and

chloride conductance have been documented in myotonic dystrophy, Charlet and co-workers investigated whether SCN4A and CLCN1 gene products are misregulated in the disease (Charlet et al., 2002; Franke et al., 1990; Koty et al., 1996). Their study showed, that similar to cTNT and the IR, the Clc-1 mRNA was aberrantly spliced resulting in 3 defective isoforms containing a premature termination codon (Fig. 5b) (Charlet et al., 2002; Mankodi et al., 2002). Using a myotonic dystrophy mouse model it was determined that loss of Clc-1 function was sufficient to induce myotonia (Mankodi et al., 2002).

A common mechanism binds the processing of the IR, cTNT and Clc-1: they are all natural targets for the alternative splicing regulator CUG-BP (Fig. 6) (Ladd et al., 2001). CUG-BP has been shown to bind U/G-rich motifs in introns adjacent to splice sites in all three of these pre-mRNAs (Charlet et al., 2002; Philips et al., 1998; Savkur et al., 2001). Furthermore, overexpression of CUG-BP in normal cells induces the same defective alternative splicing as that seen in myotonic dystrophy patients (Faustino and Cooper, 2003). The exact mechanism by which CUG-BP accumulates in the nucleus of myotonic dystrophy cells is unknown. Two possibilities have been put forward to explain this observation: the first is the haplo-insufficiency or complete lack of DMPK favours the hypophosphorylated form of CUG-BP that is shown to accumulate in the nucleus (Philips et al., 1998; Roberts et al., 1997). The second explanation suggests that double strand RNA binding proteins (dsRNA-BP) attach themselves to the CUG expansion hairpin loop thereby forming the characteristic foci and consequently inducing an elevation in CUG-BP steady-state levels in myotonic dystrophy cells (Charlet et al., 2002; Faustino and Cooper, 2003; Miller et al., 2000; Timchenko et al., 2001a).

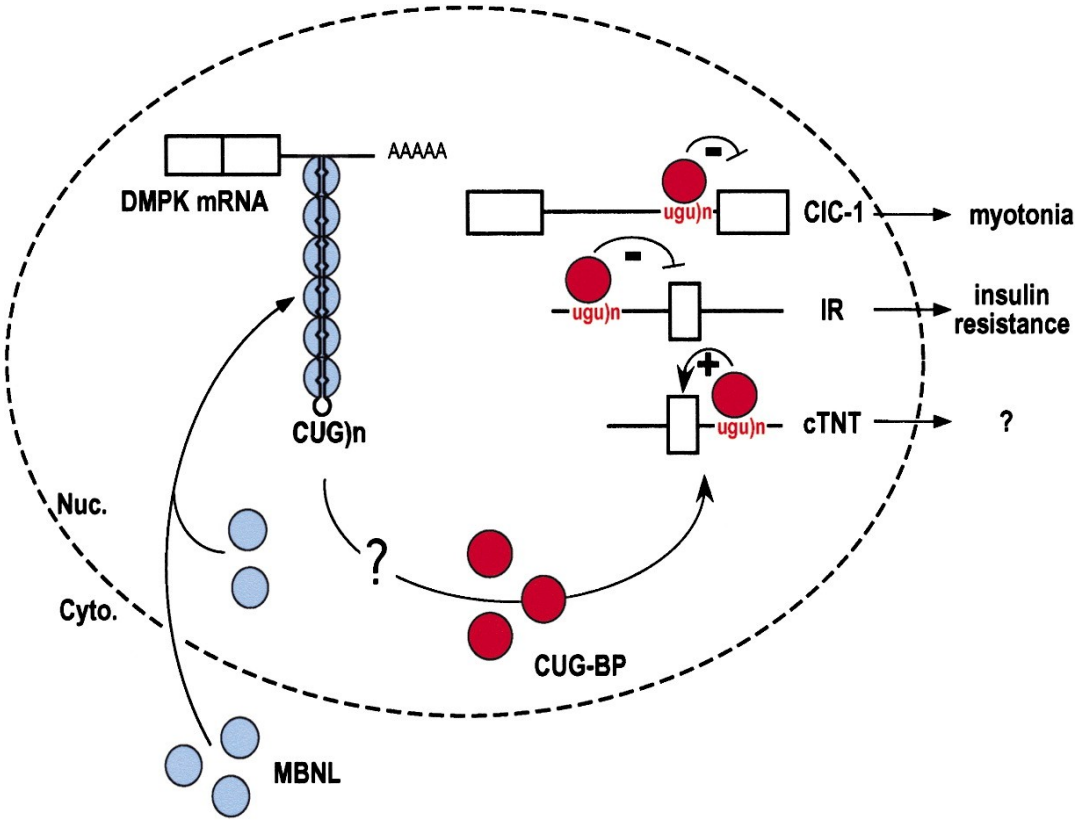

Figure 6. Model for CUG-BP-mediated alternative splicing.

Binding of MBNL on hairpin structures of the repeat tract induces an elevation in the nuclear levels of CUG-BP. The splicing factor, CUG-BP, then binds to specific sequences in target genes responsible for alternative splicing. CUG-BP can then either induce exon inclusion (cTNT) or exclusion (IR and CLC-1). Defective alternative splicing will result in several of the hallmark features of DM1.Illustration from (Faustino and Cooper, 2003)

1.1.4.6. Double-stranded CUG repeat-binding proteins

Since the discovery that mutant DMPK mRNA is fully transcribed and forms hairpin structures that are retained in the nucleus, much effort has been deployed to identify possible CUG-binding proteins (Michalowski et al., 1999; Taneja et al., 1995; Timchenko et al., 1996b). Identification of such proteins will give insight in the composition of nuclear foci that are now believed to be the cornerstone of the molecular defects involved in

myotonic dystrophy. Four CUG binding protein candidates were identified from i*n vitro* binding studies: CUG-BP, PKR, ETR-3 and MBNL (Lu et al., 1999; Miller et al., 2000; Tian et al., 2000; Timchenko et al., 1996b). Only MBNL and certain of its family members have been shown to bind long double-stranded CUG repeats and co-localize with mutant DMPK mRNA-induced foci *in vivo* (Fardaei et al., 2002; Miller et al., 2000; Squillace et al., 2002). MBNL is a mammalian homologue of the *Drosophila* muscleblind (Mbl) protein essential for the terminal stages of muscle and photoreceptor differentiation (Begemann et al., 1997). MBNL is induced by myoblast differentiation in mice and is expressed in blood, eye, cardiac and skeletal muscle (Miller et al., 2000). Because of its involvement in muscle and eye development, cellular depletion of MBNL through binding to mutant DMPK mRNAs in the nucleus in myotonic dystrophy cells may provide some explanation to the myopathy and cataracts seen in the disease.

1.1.4.7. Alteration of muscle differentiation

Myoblast differentiation is regulated by a sequential cascade of gene activation following the initial mitogen withdrawal (Fig. 7). Myoblasts are held in proliferation by presence of growth factors and of proteins Notch, Msx-1 and the bone morphogenic protein 4 (BMP-4), that block MyoD and Myf5 expression (Bailey et al., 2001). Transcription factors MyoD and Myf5 are the first genes to be activated since they are responsible for all downstream activation events in myogenesis. They bind the promoters of muscle-specific genes and activate their transcription (Rudnicki et al., 1993). Myogenin expression is the next key factor to be expressed and coincides with commitment of myoblasts to terminal differentiation (Sabourin and Rudnicki, 2000). Myogenin expression is crucial for fusion of myoblasts into multinucleated myotubes (Wright et al., 1989). Shortly following myogenin, the inhibitor p21 is expressed and marks the irreversible withdrawal from the cell cycle followed by expression terminal differentiation markers like myosin heavy chain (MyHC) and muscle creatine kinase (CK) (Sabourin and Rudnicki, 2000).

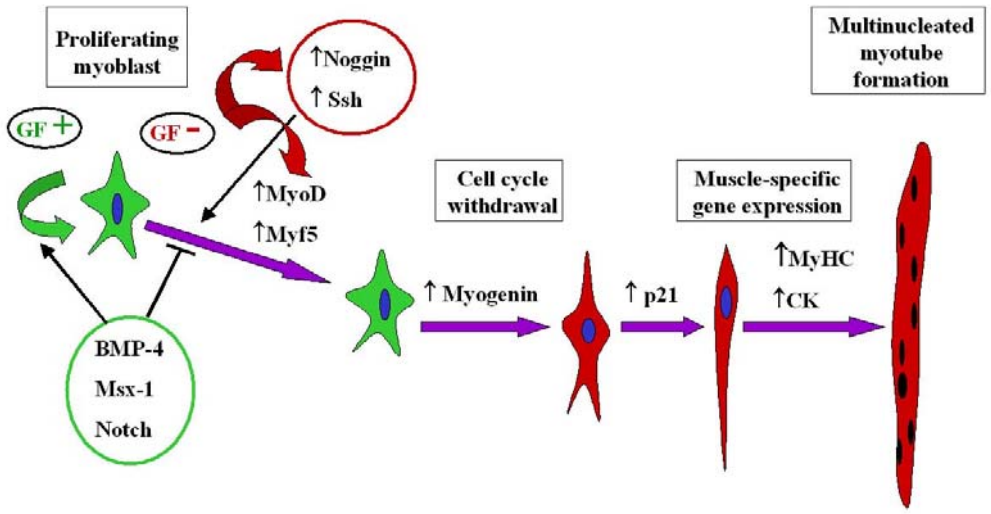

Figure 7. Temporal events of myogenesis.

Myoblasts are maintained in proliferation by presence of growth factors and through positive regulation from proliferation factors BMP-4, Msx-1 and Notch. Once growth factors are withdrawn, MyoD and Myf5 are expressed and antagonists Noggin and Ssh inhibit expression of BMP-4, Msx-1 and Notch. Master genes MyoD and Myf5 activate muscle-specific genes needed for entering the differentiation program. Myogenin is activated and triggers terminal differentiation followed by p21 that irreversibly withdraws the myoblasts from the cell cycle. Muscle-specific structural genes and markers are then expressed to allow cell fusion of myotubes formation.

Patients with myotonic dystrophy display progressive muscle weakness and wasting but in congenital cases, skeletal muscle biopsies display defects in muscle maturation indicating that alterations in myogenesis occurred during development (Harper, 2001). Myogenic satellite cells from myotonic dystrophy patients and murine C2C12 myogenic cells transfected with a plasmid expression 200 CTG repeats both show severe alterations in differentiation capabilities (Amack et al., 1999; Bhagwati et al., 1999; Furling et al., 2001a). Although it is clear that the repeat tract in the RNA has a role to play in altering

myogenesis, the exact mechanism is unclear and still strongly debated. Three different groups propose three different pathways to explain the disruption of myoblast differentiation in myotonic dystrophy. The first group used a C2C12 cell model to express various constructs containing either a CTG expansion with or without DMPK 3'UTR (Amack and Mahadevan, 2001; Amack et al., 1999). What they observed was that although the RNA containing the CUG expansion did indeed form foci in this cell model, the repeat tract alone was not sufficient to alter differentiation (Amack and Mahadevan, 2001). When the repeat tract was expressed with DMPK 3'UTR, the authors noted that myoblasts differentiation was halted in parallel to diminished expression of both myogenin and p21 mRNAs. While they did not measure any reduction in MyoD or Myf5 mRNAs in this initial study, three years later, they showed that MyoD protein levels were reduced in myoblasts expressing DMPK 3'UTR and the CTG repeat tract (Amack et al., 2002). Reduction of MyoD levels is post translational and the authors speculate that reduced protein stability may be caused by a trans effect of RNA foci (Amack et al., 2002). Interestingly, they also found that MyoD or myogenin overexpression in these cells contributed to restore myoblast differentiation. This involves that differentiation defects lies at or before myogenin expression and that events downstream are intact and functional (Amack et al., 2002).

In vitro studies by another group found that p21 was reduced in relation to defective expression of cytoplasmic CUG-BP (Timchenko et al., 2001b). They state that cytoplasmic CUG-BP induces p21 levels during myoblast differentiation by regulating its translation (Timchenko et al., 2001b; Timchenko et al., 1999). In myotonic dystrophy, CUG-BP aberrantly accumulates in the nucleus whereby being unavailable for binding to p21. Lending support to this model, Khajavi and colleagues noted that lymphoblasts with large CTG expansions had a growth advantage over cells with smaller expansions (Khajavi et al., 2001). They found that this event was due to increased Erk1,2 activation. Erk1,2, kinases involved in the regulation of meiosis, mitosis and postmitotic functions in differentiation, are negatively regulated by p21, itself downregulated in myotonic dystrophy (Johnson and Lapadat, 2002; Khajavi et al., 2001).

While all of these reports describe downstream events that may help explain why differentiation is compromised, the cause of the disturbances seem to funnel to the RNA retention and sequestration of binding proteins. While Amack and colleagues confer to these foci the capacity of inducing by some unknown mechanism the degradation of MyoD, Timchenko and co-workers attribute accumulation of the nuclear form of CUG-BP to interaction with CUG-binding proteins (Amack et al., 2002; Timchenko et al., 2001b). One recent report described that constitutive expression the muscleblind-related protein CHCR shown to accumulate in myotonic dystrophy foci can cause downregulation of myogenin and MyHC (Fardaei et al., 2002; Squillace et al., 2002). This adds new credit to the toxic gain-of-function theory through which aberrant nuclear accumulation and stabilisation of CUG-binding proteins involved in terminal differentiation of muscle may indirectly be responsible for myopathy in myotonic dystrophy.

1.1.4.8. Effects on other genes and DNA methylation

Trinucleotide CTG repeats in DNA form stable hairpin structure *in vivo* (Freudenreich et al., 1997; Mariappan et al., 1996). The chromatin structure in proximity of the repeat tract is highly perturbed and anomalously condensed from tight nucleosome assembly (Wang et al., 1994). This highly condensed region of the chromatin could result in transcription repression (Wang et al., 1994). In fact, the DMPK locus is flanked by two genes: upstream by DMWD and downstream by SIX5 (formerly known as DMAHP) (Fig. 8) (Alwazzan et al., 1999; Klesert et al., 1997). Both these genes have been shown to be downregulated in presence of large CTG expansions (Alwazzan et al., 1999; Klesert et al., 1997; Thornton et al., 1997). While the role of the DMWD gene product is suspected to be involved in brain-related symptoms in myotonic dystrophy, deficiency of SIX5 causes cataracts in knockout mouse models (Klesert et al., 2000; Sarkar et al., 2000; Westerlaken et al., 2003). While these results support a *cis* effect for the DM mutation, these mouse models did not present any muscle-related abnormalities (Klesert et al., 2000; Sarkar et al., 2000).

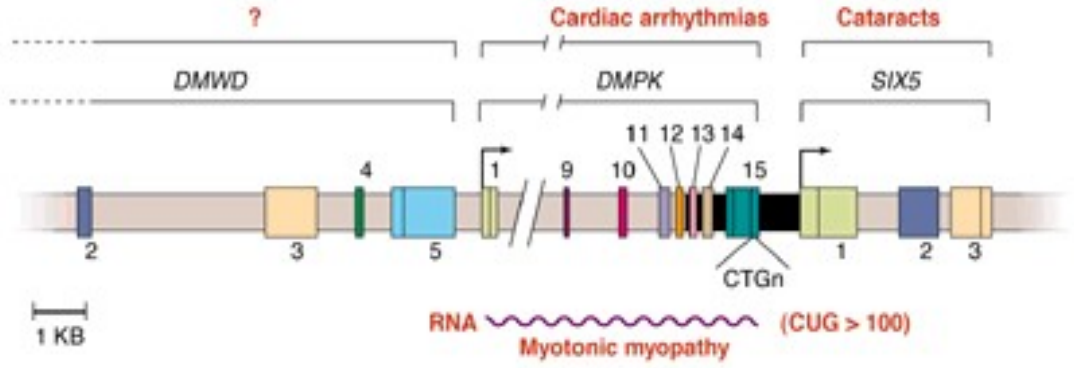

Figure 8. Diagram of DMPK and adjacent genes at the DM1 locus.

The DMPK gene is flanked by DMWD and SIX5 genes. The DMPK polyadenylation site is less than 300 bp from the SIX5 initiation codon. In black are represented CpG islands that are methylated in congenital myotonic dystrophy (Image from (Tapscott, 2000).

While the triplet repeats may constitute themselves a regulatory element for adjacent genes, the CpG island at the 3' end of the DMPK gene may also have a potential role in gene regulation (Boucher et al., 1995; Steinbach et al., 1998). In fragile X syndrome, hypermethylation of the CpG island in the promoter of the FMR1 gene causes transcriptional repression (Knight et al., 1993; Schwemmle et al., 1997). A study on the methylation status of this CpG island revealed that although there is no allele-specific methylation or parental imprinting (Jansen et al., 1993; Shaw et al., 1993), hypermethylation was present in cells from congenital myotonic dystrophy patients (Steinbach et al., 1998). Hypermethylation of the CpG island in the DMPK gene constitutes one of the only molecular alterations to clearly distinguish adult from congenital forms of myotonic dystrophy in primary culture cells (Filippova et al., 2001; Steinbach et al., 1998).

Unlike its role in fragile X syndrome, hypermethylation of the CpG island in 3' of the DMPK gene has been shown to disrupt the function of an insulator allowing delimitation between DMPK and SIX5 *cis*-acting elements (Filippova et al., 2001). An insulator is a gene boundary element that blocks an enhancer from acting on a promoter when placed between them or by protecting a transgene from chromatin silencing when placed at either end (Zhan et al., 2001). Essentially, their role is to prevent positioning effects thereby allowing independent function and regulations of the genes within their boundaries (Zhan et al., 2001). CTCF is the zinc-finger protein that can bind insulator sequences to confer functionality (Bell et al., 1999). Two CTCF binding sites flank the CTG tract in the DMPK gene to form a functional insulator (Fig. 9) (Filippova et al., 2001). CTCF proteins cannot bind the insulators when CpG islands are hypermethylated. This causes the loss of insulator activity and may allow crosstalk between the Six5 enhancer and the DMPK promoter.

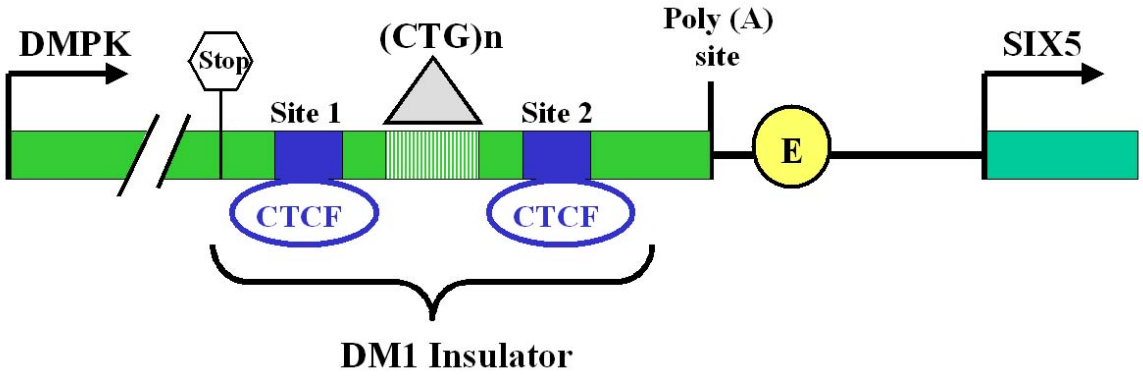

Figure 9 DM1 Locus showing positioning of CTCF binding sites.

CTG repeats are flanked by 2 CTCF binding sites. All three elements are needed for enhancer-blocking activity. CTCF binding sites 1 and 2 are within CpG islands that are hypermethylated in congenital myotonic dystrophy. Hypermethylation of the CpG islands prevent binding of CTCF, thus abolishing insulator activity thereby exposing the DMPK promoter to cis effects from the Six5 enhancer (E).

1.1.5. Myotonic dystrophy type 2

Since the discovery of the DM mutation, genetic testing has been used to validate clinical diagnosis for myotonic dystrophy. These tests revealed that 98% of patients who displayed clinical symptoms for myotonic dystrophy had in fact the DM mutation (Mahadevan et al., 1992). However, between 1-2 % of patients that displayed most of the classical symptoms of myotonic dystrophy did not have the characteristic CTG expansion in 3'UTR of the DMPK gene (Mahadevan et al., 1992).

The first clinical evidence that there might exist a second form of myotonic dystrophy came from patient studies in 1994 (Ricker et al., 1994). Patients in these studies displayed most clinical symptoms of myotonic dystrophy without the CTG expansion in the DM1 locus. They also displayed proximal muscle weakness as opposed to distal as in classical myotonic dystrophy. This distinct form of myotonic dystrophy was from then on named proximal myotonic myopathy (PROMM), until the molecular identification of the mutation in 2001 by Liquori and colleagues (Harper, 2001). There are five main clinical symptoms that distinguish myotonic dystrophy and PROMM (Harper, 2001; Vihola et al., 2003):

1. **The predominant distributions of muscle weakness is proximal in PROMM**
2. **PROMM is associated with muscle pain**
3. **PROMM patients display type II fiber atrophy as opposed to type I**
4. **Facial weakness is absent in PROMM**
5. **No CNS involvement has been documented for PROMM**

These findings quickly led research teams in a rally to identify a second DM locus. In 1998, a second myotonic dystrophy locus was mapped to a 10-cM region on chromosome 3 by studying a five-generation family showing symptoms of a 'myotonic dystrophy-like' disease (Ranum et al., 1998). Three years later, the mutation responsible for the unaccounted cases of classical myotonic dystrophy was coined (Liquori et al., 2001). PROMM or myotonic dystrophy type 2 (DM2) is caused by a CCTG expansion in intron 1

of the zinc finger protein 9 (ZNF9) gene (Liquori et al., 2001). A normal individual has less than 70 repeats, while severely affected individuals can have more than 11000 of the tetranucleotide repeats (Liquori et al., 2001). As seen in myotonic dystrophy type 1, i*n situ* hybridization with a fluorescent CAG probe revealed that RNA harbouring large CCUG expansions can also form discrete foci in the nucleus of DM2 cells and bind muscleblind proteins (Fardaei et al., 2002; Liquori et al., 2001; Mankodi et al., 2001). However, there is some preliminary evidence indicating that the CCUG expansion is spliced out from the mutant ZNF9 pre-mRNA and exists in the nucleus as a stable lariat (L. Ranum, unpublished data, Glasgow 2003). In whole, these results support that DM2 is caused by the same gain-of-function theory as proposed for myotonic dystrophy type I, where an RNA exerts toxicity by forming nuclear foci through the recruitment of CUG-binding proteins. Differences between these two forms of myotonic dystrophy may lie within *cis* or *trans* effects caused by the expansions in the DNA and not the RNA (L. Ranum, unpublished data, Glasgow 2003).

1.1.6. Molecular pathogenesis model for myotonic dystrophy

The great heterogeneity of symptoms and defects at the molecular, cellular and metabolical level make myotonic dystrophy an extremely complicated disease to understand. Identification of the mutation in 1992 only confirmed that understanding the basis of the disease was still a long way down the road. There is now a general consensus that multiple factors contribute to the disease. Three theories have been put forward over the past 5 years to give some explanation on how triplet expansions in the untranslated region of a gene can cause myotonic dystrophy (Tapscott, 2000; Tapscott and Thornton, 2001):

1) **The expanded CTG repeats cause haplo-insufficiency of the DMPK protein by nuclear retention of its mRNA**

2) **Modification of the regional chromatin alters adjacent gene expression**

3) **Aberrant sequestration of CUG-binding proteins leads to toxic foci formation and aberrant mRNA splicing**

Much of this three-level theory is based upon results generated with the three major mouse models:

1) **DMPK knock-in/out mice (Jansen et al., 1996)**

2) **Transgenic mice expressing CTG repeats in 3'UTR of the human actin or DMPK gene (Mankodi et al., 2000; Seznec et al., 2000)**

3) **SIX5 knock-out mice (Klesert et al., 2000; Sarkar et al., 2000)**

However, some new reports in the literature have been breaking-down these long-standing beliefs. For instance, how can the DM2 mutation, located on a completely different chromosome, generate most of the same clinical symptoms as myotonic dystrophy type I? Involvement of the CTG repeats in the disruption of neighbouring gene expression (DMPK, Six5 and DMWD) is now though to be irrelevant, or at very best of meek influence in disease physiopathology. Second, spinocerebellar ataxia type 8 (SCA8) which is caused by an expanded CTG repeat that is transcribed, generates a completely different clinical portrait as compared to myotonic dystrophy types 1 or 2 (Koob et al., 1999; Nemes et al., 2000). The answer to this riddle may reside in tissue-specific gene expression, as SCA8 is most predominantly expressed in the brain (Koob et al., 1999). Further credibility may be provided by the fact that DMPK and not ZNF9 is expressed in the brain, and only myotonic dystrophy type I patients, especially those suffering from the congenital from, exhibit strong CNS involvement and mental retardation, as seen in SCA8 (Harper, 2001; Nemes et al., 2000).

So as of late 2003, it is believed that the main driving force in generating myotonic dystrophy clinical symptoms is the toxic RNA gain-of-function theory based on sequestration and aberrant function of critical CUG-binding proteins (Mankodi and Thornton, 2002). This theory must now also be fine-tuned in specifying that the RNA containing the large CUG or CCUG repeats be expressed in smooth, skeletal and cardiac

muscle to provoke the necessary disruptions to generate the typical clinical symptoms. Myotonic dystrophy is the first example of a dominant disease caused by a toxic gain-of-function mechanism of a mutant RNA.

1.1.7. Therapeutic strategies for myotonic dystrophy

Since it is now clear that myotonic dystrophy type I is caused by a toxic mRNA, what are the therapeutic approaches available? While certain features of the disease phenotype such as the myotonia, arrhythmias and daytime drowsiness are treated today with drugs, surgery for other features such as cataracts and ptosis has been successful (Harper, 2001). Nonetheless, treatment of symptoms is only provisional since the disease is of neurodegenerative nature and worsens with time. A successful RNA-based gene therapy may be the one of the most effective ways at this time to attenuate symptoms of the disease.

As it has been mentioned previously, the mutant DMPK mRNA may be accountable for the great majority if not all of the muscle-related clinical symptoms seen in adult onset myotonic dystrophy. An ideal gene therapy for myotonic dystrophy would specifically target and destroy the nuclear-retained mutant DMPK.

Part II: Molecular tools for potential therapeutics

1.2. Nucleic acid-based gene silencing

Ever since the first demonstration that antisense oligodeoxynucleotide (AS-ODN) could silence gene expression more than 25 years ago, hope was borne for developing specific targeting strategies for research and therapeutic use (Stephenson and Zamecnik, 1978). Several tools now exist to knock-down the expression of specific genes. Virtually all these tools are comprised of nucleic acids or nucleic acid analogs capable of base-pairing in a Watson-Crick fashion with a target sequence on either DNA or RNA (Fig. 10). Modification of nucleic acids confer increased resistance to nucleases, provide different affinities for either DNA or RNA and/or increase specificity for a given target. Most tools developed today target and bind to the mRNA of a specific gene resulting in post-transcriptional gene silencing (PTGS) (Table 5).

There are three main pathways whereby these molecules can induce PTGS by targeting the RNA (Kurreck, 2003):

1. **They can catalytically cleave the target RNA in absence of accessory proteins (e.g. ribozymes)**

2. **They induce a protein-dependent degradation of a specific RNA once bound to their target (e.g. AS-RNAs, AS-ODNs, siRNAs)**

3. **Block the translation of the mRNA by preventing protein binding (e.g. miRNAs, AS-RNAs, AS-ODNs)**

Figure 11 illustrates four examples of PTGS. Despite that all these molecules are capable of PTGS, their respective mechanisms of action are very different. Fine-tuning these tools for specific applications and organisms requires in depth knowledge of their functions. The

following sections will provide an overview of the most commonly utilised PTGS tools and focus on their mechanism in mammalian cells.

Table 5 Post-transcriptional silencing tools

Tools	Mechanism
Antisense oligodeoxynucleotide (**AS-ODN**)	Induction of RNAse H
Antisense RNA (**AS-RNA**)	Protein-dependent cleavage
2'-O-methyl phosphorothioate antisense oligodeoxynucleotide (**AO**)	Inhibition of splicing (Exon skipping)
DNAzymes	Protein-independent catalytic cleavage
External guide sequences (**EGS**)	RNAse-P mediated cleavage
Morpholino antisense (**MF-AS**)	Inhibition of translation
Micro RNAs (**miRNA**)	RNAi (inhibition of translation)
Peptide nucleic acids (**PNA**)	Inhibition of translation
Phosphorothioate oligodeoxynucleotide (**PS-ODN**)	Induction of RNAse H
Ribozyme (**RBZ**)	Protein-independent catalytic cleavage
Small interfering RNA (**siRNA**)	RNA interference (RNAi) (cleavage)
Small hairpin RNA (**shRNA**)	RNAi (cleavage)

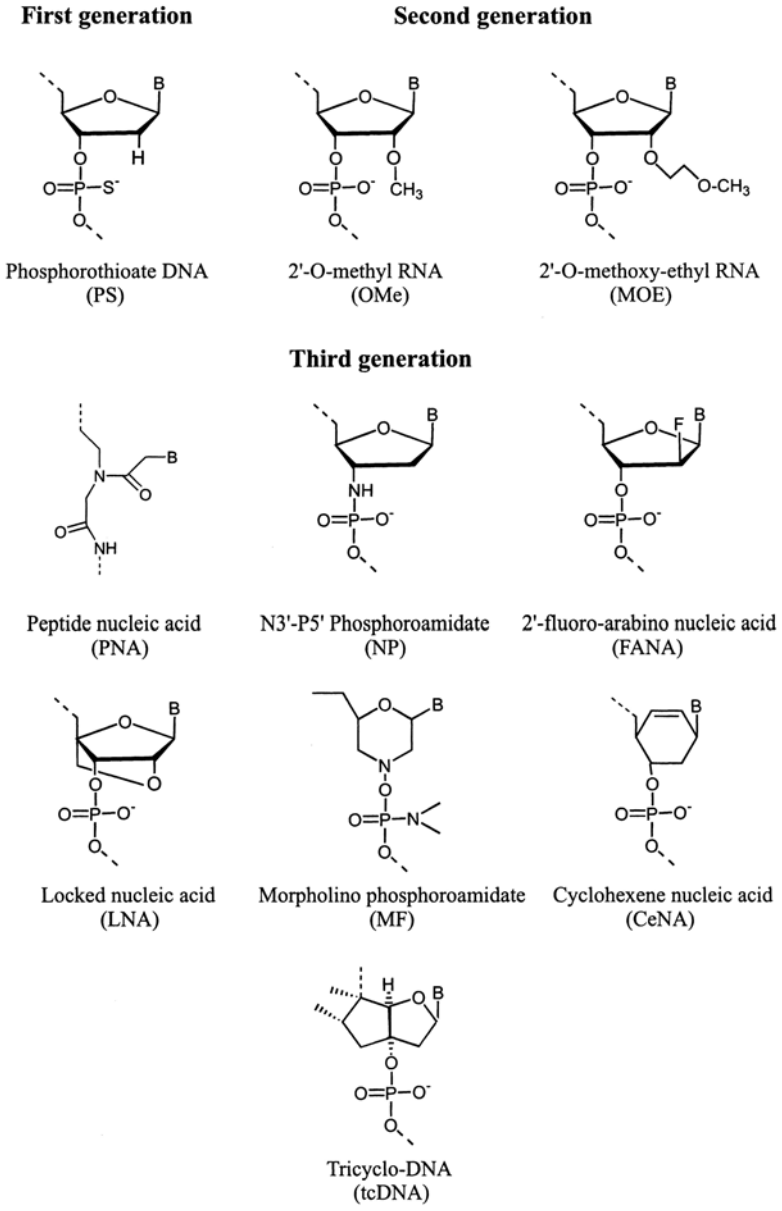

Figure 10 Nucleic acid analogs

Three generations of nucleic acid analogs capable of binding RNA or DNA. First generation analogs add a sufur to the non-bridging oxygen atom of the phosphodiester bond conferring additional stability. Second generation analogs add alkyl modifications at the 2' position of the ribose which avoids stimulating RNAse H activity when bound to an RNA. Third generation analogs add substantial modifications to the sugar and/or to the phosphate backbone conferring resistance to DNAses. B denotes one of the bases adenine, guanine, cytosine or thymine. (Illustration from (Kurreck, 2003)).

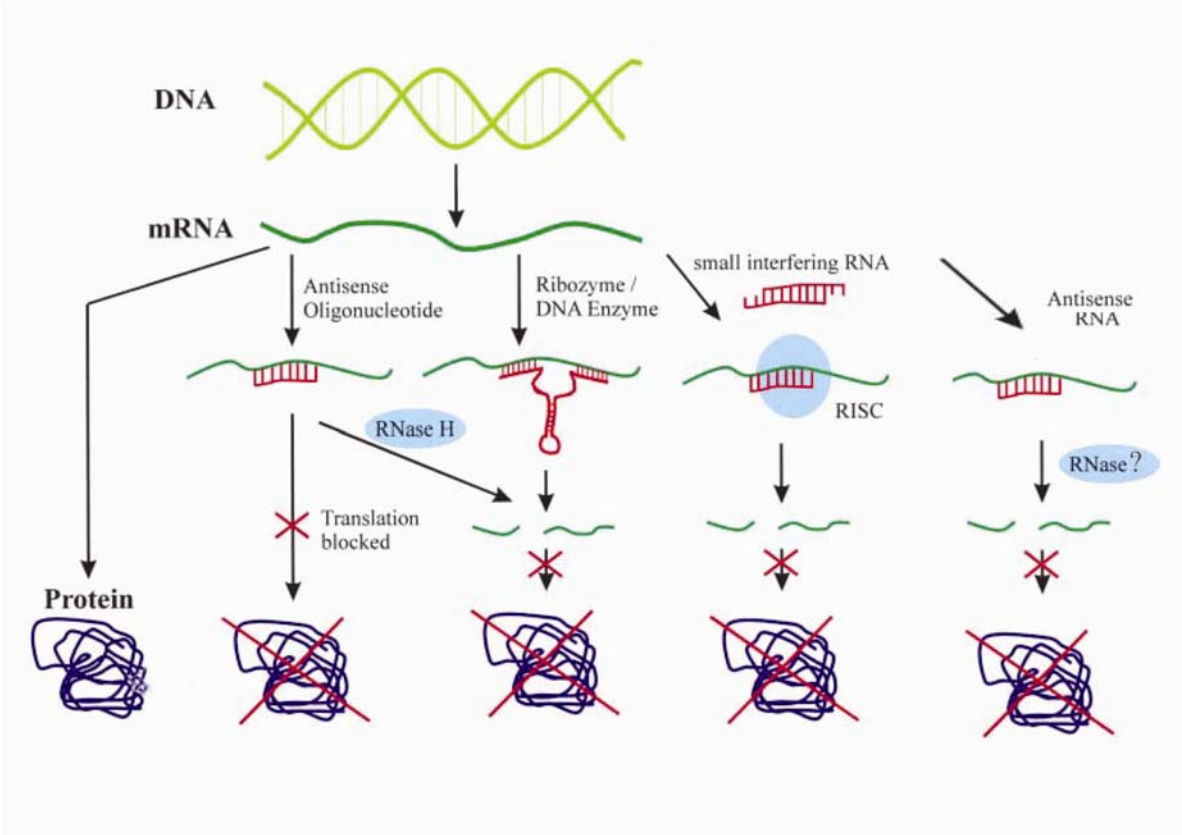

Figure 11 Examples of post-transcriptional gene silencing tools

Four common approaches for targeting mRNA to induce PTGS and their correspond mechanism of action. (Illustration adapted from (Jen and Gewirtz, 2000; Kurreck, 2003))

1.2.1. Antisense ODNs

Antisense ODNs are the oldest tools to either disrupt gene transcription or induce PTGS. They are still today the cheapest and fastest method for determining gene function and for target validation. Depending on their length and sequence, they can be designed to either target RNA or DNA.

There exists two generalized approaches to specifically target and disrupt genes using ODNs: homologous recombination and inhibition of gene transcription by triple-helix

formation (Camerini-Otero and Hsieh, 1995; Jen and Gewirtz, 2000). Both methods employ the use of long antisense oligodeoxynucleotides (AS-ODN) complementary to a genomic sequence. Although homologous recombination is time-consuming and very inefficient in primate cells, it remains the standard for generating transgenic mice (Jen and Gewirtz, 2000). Homologous recombination is produced basically by introducing in target cells an ODN containing a selection marker in the midst of sequences complementary to the target gene. The ODN is integrated following a crossover event during cell division and the target gene is thus inactivated (Fig 12). The other method employed to block transcription utilises long AS-ODN targeting a specific gene. The AS-ODN base-pars with its target sequence and dislodges the complementary DNA strand (Camerini-Otero and Hsieh, 1993). This results in the formation of a triple helix structure that prevents transcription from initiating (Fig 12).

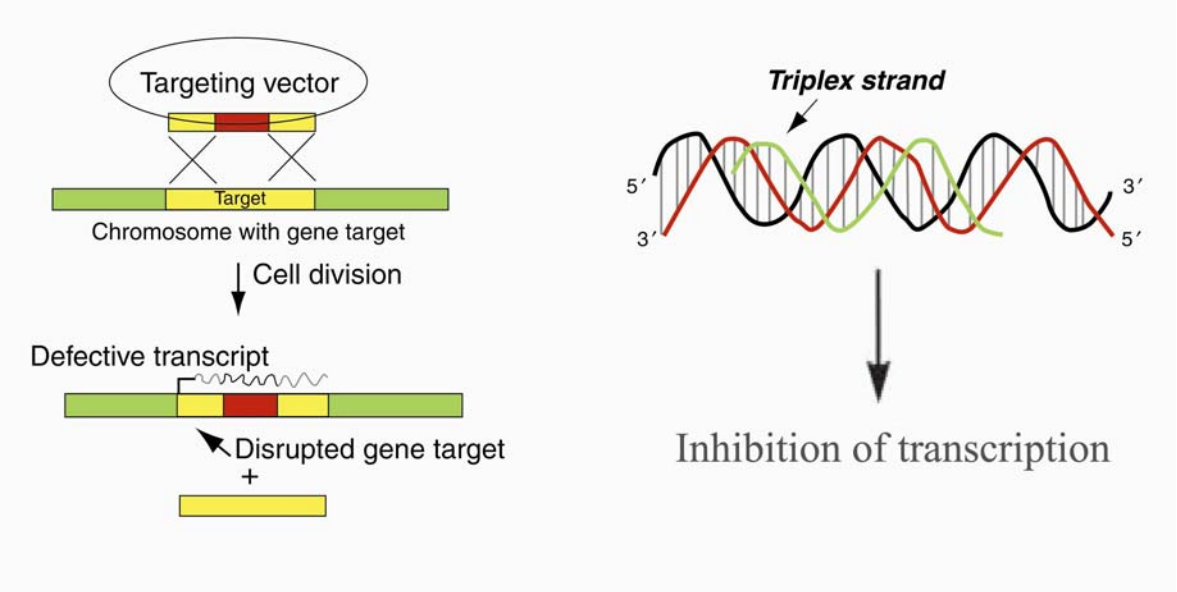

Figure 12 Mechanisms for gene knock-down using long AS-ODNs

DNA targeting methods. Right panel shows an example of gene disruption by homologous recombination, the left panel shows transcription inhibition caused by triplex formation following AS-ODN binding. (Illustration adapted from (Jen and Gewirtz, 2000; Kurreck, 2003))

Antisense ODNs can also be designed to target RNA. Generally these are short stretches of 15-30 nucleotides. Phosphorothioate AS-ODNs have enhanced nuclease resistance with a half-life in human serum of approximately 9-10 hours compared to less than 1 hour for unmodified AS-ODNs (Campbell et al., 1990). It is still not fully understood today how AS-ODNs induce PTGS or whether this occurs in the cytoplasm or the nucleus (Jen and Gewirtz, 2000). Because target sequences are not equally accessible due to strong secondary structures and protein binding to the endogenous RNA, identification of available binding sites is necessary to achieve maximum results (Scherr et al., 2000a; Scherr and Rossi, 1998; Scherr et al., 2000b). Although AS-ODNs binding to mRNA may play a role in inhibiting translation initiation, its main mode of action resides in recruiting cellular RNAse H. DNA-RNA duplexes are short-lived in live cells and in cells extracts because they are strong inducers of RNAse H activity that hydrolyses the RNA in the duplex (Cazenave et al., 1989). Using second generation AS-ODN analogs with 2' modifications to the ribose will increase DNA-RNA stability but abolishes RNAse H activity (Jen and Gewirtz, 2000). PTGS will then occur only through translation inhibition or possibly defects in RNA processing and export due to DNA-RNA duplex formation in the nucleus. The main caveats in using AS-ODN in developmental studies or for gene therapy is that their administration can be toxic causing undesireable side-effects, and target RNA is continuously synthesised thus requirering frequent re-administration of the AS-ODNs (Heasman, 2002).

Despite the downfalls of using AS-ODNs, two studies have shown that DMPK mRNA levels are reduced when mouse myoblasts were treated with PS AS-ODNs *in vitro* (Galderisi et al., 1996; Melone et al., 1998). Unfortunately, this method was not specific, as it targeted indiscriminately both mutant and normal DMPK transcripts. AS-ODNs are incapable of distinguishing alleles based upon a single nucleotide polymorphism that would allow specific targeting of mutant DMPK mRNA. This is why it is unconceivable to develop an AS-ODN-based therapy for myotonic dystrophy.

1.2.2. Antisense RNAs in mammalian cells

Formation of double-stranded RNA (dsRNA) is a naturally occurring phenomenon in both prokaryotes and eukaryotic cells. Double-stranded RNA formation has different roles and consequences depending on the length of the RNA-RNA duplex, whether the duplex is interrupted by mismatches or not and whether it is located in the nucleus or the cytoplasm (Kumar and Carmichael, 1998; Scadden and Smith, 2001). In general, small RNA duplexes (<30 bp) have physiological roles in mammalian cells by regulating gene expression through the RNAi pathway that will be discussed later on, as opposed to long duplex RNAs (>30) that elicit potent antiviral responses when they are present in the cytoplasm (Saunders and Barber, 2003; Scadden and Smith, 2001). Figure 13 illustrates four dominant pathways that are activated when long dsRNAs are detected in mammalian cells. Several proteins such as adenosine deaminases that act on RNA (ADARs), protein kinase R (PKR), 2',5' Oligoadenylate (OligoA) synthetase and RNAse III family members are capable of binding to dsRNA and trigger a specific response to it.

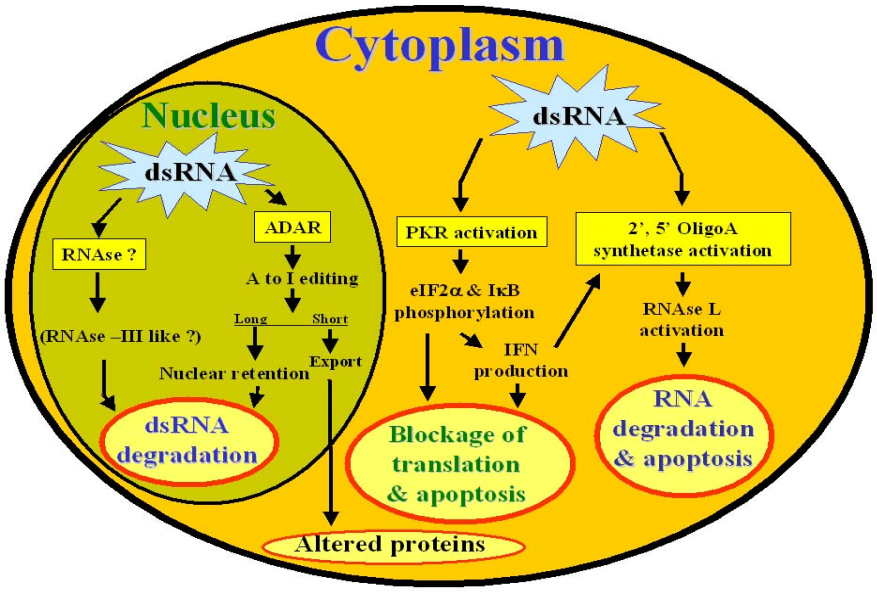

Figure 13 Consequences of long dsRNAs in the nucleus and cytoplasm of mammalian cells

Antisense RNA within the cytoplasm can activate several pathways. Two major pathways implicate PKR and 2',5' oligoA synthetase activation. PKR is activated through binding to

dsRNA. Activated PKR phosphorylates eIF2α and IκB which results in translation blockage and IFN secretion respectively. Activated 2',5' oligoA synthetase generates oligoadenylates which in turn activate RNAse L. RNAse L can degrade both cellular and viral ssRNAs and dsRNAs. General nuclear pathways involve ADAR activation and RNAse-III-like induction. Long dsRNAs (>100 bp) that activate the ADAR protein family will be deaminated on A residues and completely unwound. These RNAs will be retained in the nucleus and degraded. Short dsRNAs (30-100 bp) will only be partially deaminated, exported to the cytoplasm and can be translated into altered proteins since I's are decoded as G's.

Cellular responses are very different depending on whether dsRNA is detected in the cytoplasm or the nucleus. Double-strand RNA in the cytoplasm is often reminiscent of a viral infection and is addressed by a rapid and potent response. General mechanisms in the cytoplasm include the PKR and 2',5'OligoA synthetase pathways (Kumar and Carmichael, 1998). PKR is activated when bound to dsRNA. PKR has two important phosphorylation substrates which are eukaryotic initiation factor 2α (eIF2α) and IκB (Kumar et al., 1994). Un-phosphorylated IκB binds to NFκB and blocks its nuclear localizations signal. When IκB is phosphorylated, the complex dissociates and NFκB translocates to the nucleus and activates related genes (Kumar et al., 1994). One of these genes codes for interferon β (IFN β). IFN−β production commences a cascade which causes blockage of protein and RNA synthesis and can ultimately lead to cell cycle arrest and apoptosis if secretion is sustained (Kalvakolanu and Borden, 1996). In parallel, phosphorylated eIF2a inhibits eIF-2B which is responsible to catalyze the guanine nucleotide exchange factor reaction required for initiating protein synthesis (Samuel, 1993). As mentioned previously, dsRNA activates 2',5'OligoA synthetase but this enzyme is also activated as a downstream event of IFN production (Castelli et al., 1997; Kumar and Carmichael, 1998). 2',5'OligoA synthetase polymerises ATP with 2', 5' linkages. 2',5' adenylates in turn activate RNAse L that degrades dsRNA but also single-strand RNA (ssRNA). Extensive stimulation of RNAse L activity induces apoptosis (Castelli et al., 1997).

Nuclear events relating to the presence of dsRNA are much more specific and subtle, primarily because it is though that most naturally occurring AS-RNAs act within the nucleus (Kumar and Carmichael, 1998). DsRNA within the nucleus does not trigger PKR, IFN or 2',5' OligoA synthetase pathways (Kumar and Carmichael, 1998). Although there is some support that formation of dsRNA duplexes may play a role in inhibiting transcription, splicing, RNA export and even reducing RNA stability in the nucleus, there are but two mechanisms that have been described in detail: dsRNA deamination and enzymatic degradation by RNAses (Kumar and Carmichael, 1998). RNAse III or RNAse III-like (Dicer, Drosha, …) are the likely candidates for initiating dsRNA degradation in mammalian cells (Lee et al., 2003; Myers et al., 2003; Wu et al., 2000). RNAse III is normally implicated in processing pre-RNA, small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) (Filippov et al., 2000; Lee et al., 2003; Saunders and Barber, 2003). RNAse III family members have high specificity for dsRNA and introduce single-strand nicks or staggered double strand breaks in the duplex RNA (Saunders and Barber, 2003). Dicer, is an essential member of the RNAi pathway that leads to PTGS (Ketting et al., 2001; Knight and Bass, 2001). The other well known nuclear mechanism to deal with dsRNAs is hydrolytic deamination by the ADAR family of proteins (Saunders and Barber, 2003). These enzymes substitute adenosines (A) by inosines (I) on dsRNA rendering it unstable (O'Connell et al., 1995). The fate of these deaminated dsRNAs will depend only on their length (Kumar and Boriek, 2003; Saunders and Barber, 2003). Long (>100 bp) perfect duplexes are fully deaminated and are destined to be retained in the nucleus and degraded by an unidentified process (Kumar and Carmichael, 1998). Short or imperfect duplexes are partially deaminated and exported to the cytoplasm. These transcripts are then translated into altered proteins, if appropriate translation elements are present, because I's are recognised as if they were G's (Kumar and Carmichael, 1998). It is of note that when duplex RNAs are in the range of 19 to 30 bp, they will enter the RNAi pathway discussed in section 1.2.4. .

Antisense RNAs are great tools for studying gene function and they offer much potential for an eventual gene therapy. They have the advantage to be easily designed against any RNA target desired and are expressed by simple expression systems. As with AS-ODNs, finding accessible binding sites on a target RNA remains of critical concern. Nonetheless, several methods exist to identify best possible binding sites (Scherr et al., 2000a; Scherr and Rossi, 1998; Scherr et al., 2000b). For applications in myotonic dystrophy, long AS-RNAs directed against the sequestered mutant DMPK mRNA may prove effective since they would be retained in the nucleus, thus avoiding activation of PKR and interferon responses in the cytoplasm. The main drawback of this tool is that long AS-RNAs may have small regions complementary to other RNAs. Duplex formation may lead to undesired side-effects *in vivo*. Also, several other mechanisms implicated in nuclear degradation of dsRNAs are not fully understood nor identified.

1.2.3. Ribozymes

Discovery of naturally occurring catalytic RNAs in the early 1980's has revolutionized the way we perceive RNA, that is why Sydney Altman and Thomas Cech received in 1989 the Nobel Prize in Chemistry for their discovery (Cech et al., 1981; Guerrier-Takada and Altman, 1984). These RNAs named ribozymes naturally catalyze the cleavage and/or ligation of RNA by transesterification or hydrolysis of phosphate groups in lower eukaryotes, bacteria and viruses (Doherty and Doudna, 2000). Their physiological role in these organisms is to catalyze RNA processing by cleavage or ligation reactions required for splicing or for rolling circle RNA replication (Butcher, 2001; Doherty and Doudna, 2000). Group I and II intron ribozymes and the catalytic RNA component of ribonuclease P (RNAseP) are large (>100 nt) and complex RNAs that catalyze two-step self-splicing reactions (Doherty and Doudna, 2000). There exists also four smaller (30-100 nt), simpler ribozymes such as the hammerhead, hairpin, Neurospora Varkud satellite and hepatitis delta virus ribozymes. The secondary structure of a hammerhead and a hairpin ribozyme are depicted in figure 14. These small ribozymes are capable of site-specific cleavage by orienting a 2' oxygen to conduct a nucleophile attack on the adjacent phosphate in the

target RNA backbone (Doherty and Doudna, 2000). All ribozymes rely on secondary and tertiary structural folds to orient the cleavage site in the catalytic core of the ribozyme, consequently divalent metal ions such as Mg^{2+} are of particular importance in the catalytic reaction (Dahm and Uhlenbeck, 1991; Juneau and Cech, 1999; Laggerbauer et al., 1994; Piccirilli et al., 1993; Rangan et al., 2003; Sugimoto et al., 1989; Toh et al., 1987).

Ribozymes have the particularity of being catalytically active in absence of proteins except for RNAse P that requires protein cofactors for processing tRNAs (Doherty and Doudna, 2000; Frank and Pace, 1998). While ribozymes were initially identified by their self-splicing reactions, it is possible to direct a ribozyme to *trans*-cleave practically any target RNA (Phylactou et al., 1998b). Ribozymes bind to substrate RNA through Watson-Crick base-pairing thus allowing sequence-specific cleavage of target RNA. Here two ribozymes with particular relevance in gene therapy will be shortly reviewed: the small hammerhead ribozyme and the trans-splicing Tetrahymena group I ribozyme.

1.2.3.1. The hammerhead ribozyme

The hammerhead ribozyme was discovered as a self-splicing RNA molecule isolated from plant satellite viroid RNA that replicates by a rolling-circle mechanism (Cotten and Birnstiel, 1989; Symons, 1992). It can cut any NUH sequence on a target RNA, where N is any ribonucleotide, U is uracile and H is any ribonucleotide but guanine (Eckstein, 1996). Hammerhead ribozymes are critically dependent on the presence of divalent ions for both proper folding and catalytic activity (Dahm and Uhlenbeck, 1991). The hammerhead ribozyme is one of the smallest but most active *trans*-cleaving ribozymes identified. It consists of less than 40 nucleotides comprising two substrate binding arms and a catalytic domain (Fig. 14A). The length of the substrate arms may vary considerably without affecting cleavage efficiency. Longer arms will however augment ribozyme specificity and stability but reduce dissociation and turnover rates (Rossi, 1999). A consensus between 5 and 7 nucleotides for each arm is a good compromise between stability and rapid dissociation rates (Fritz et al., 2002a; Fritz et al., 2002b; McCall et al., 2000). Nucleotides located in the first loop of the catalytic core are essential for ribozyme activity.

Substitutions or deletions in this region will result in complete loss of catalytic activity (Chang et al., 2002; Hendrix et al., 1996; Lee et al., 2001; McCall et al., 2000).

1.2.3.2. The Tetrahymena group I intron ribozyme

This ribozyme was the first group I intron ribozyme identified and the best characterized (Grabowski et al., 1981). This long, 421 nucleotide ribozyme self-splices in two distinct steps (Cech, 1990). The first step consists in cutting the phosphodiester bond at the exon-intron junction; in the second step, flanking 5' and 3' exons are ligated and the ribozyme is removed (Long et al., 2003). The natural self-splicing ability of this ribozyme was been adapted for trans-splicing RNA targets, as depicted in figure 15 panels A and B (Sullenger and Cech, 1994). Basically, for trans-splicing to occur, the free ribozyme must base-pair with its target sequence in 5' of the RNA via an internal guide sequence (IGS) located upstream of the group I intron ribozyme (Fig. 15A). The only sequence requirement is the formation of a U:G base pair between the end of the 5'exon recognition site and the ribozymes' IGS (Fig. 15) (Phylactou et al., 1998b). Engineering of group I intron ribozymes was taken a step further in recent years with ribozyme-mediated repair of mutant RNAs (Fig 15C). This is done by designing a group I intron ribozyme with an IGS sequence complementary to the region upstream of the undesired mutation and a corrective exon sequence fused in 3' of the ribozyme. Several genes related to disease have been successfully repaired *in vitro* using trans-splicing repair, such as Sickle β-globin, mutant DMPK and cClC-1 mRNAs but with extremely low overall efficiency; less than 1.2 % of all target RNAs were repaired (Lan et al., 1998; Phylactou et al., 1998a; Rogers et al., 2002; Sullenger and Cech, 1994). Inappropriate folding of this complex ribozyme in the cell may partly explain why efficiency is so low (Long et al., 2003). Since it relies on complex folding of secondary and tertiary structures, all but a few ribozyme transcripts may acquire the correct conformation for catalytic activity (Long and Sullenger, 1999).

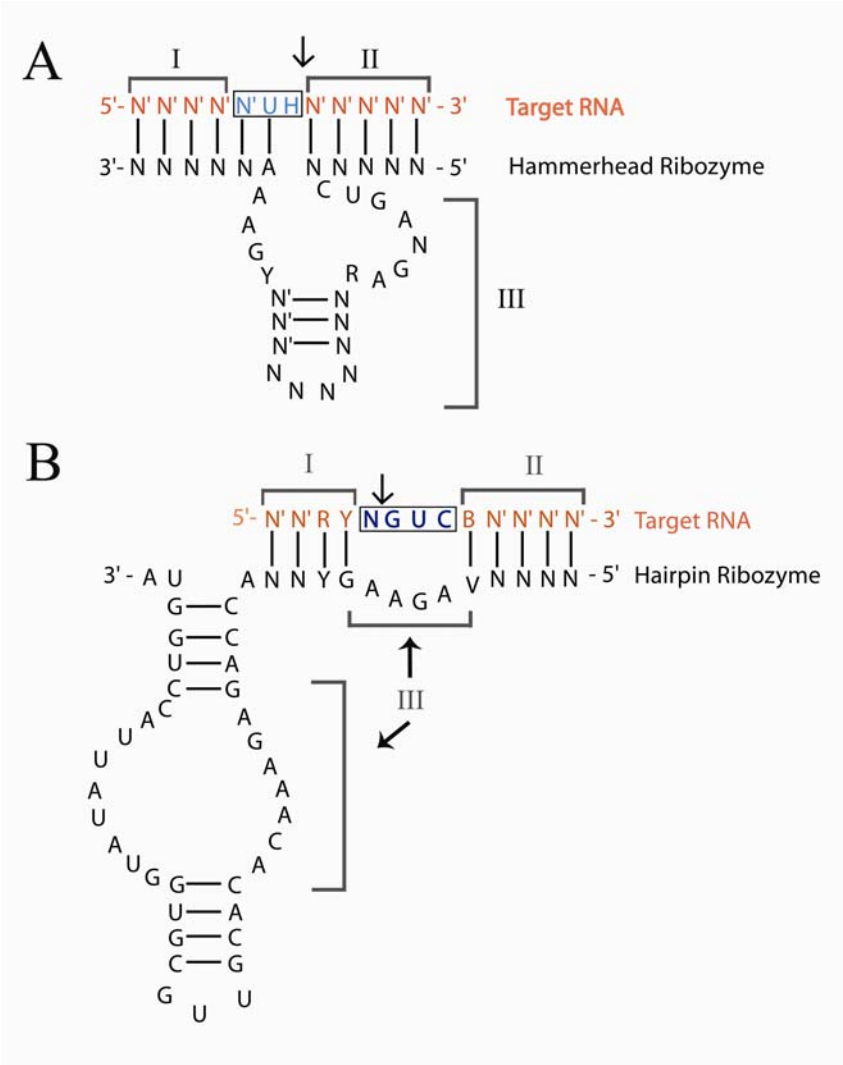

Figure 14 The hammerhead and hairpin ribozymes

Consensus cleavage sequences are indicated in blue in both panels. N represents any ribonucleotide; N' represents the corresponding complementary ribonucleotide; H, any ribonucleotide but G; Y, any pyrimidine; R, any purine; B and V, any ribonucleotide other than A and U respectively. Arrows indicate cleavage sites. ***I*** *indicates the 3' substrate arm,* ***II*** *the 5' substrate arm and* ***III*** *the catalytic domain(s) of the ribozymes.* ***(A)*** *The hammerhead ribozyme (black) and its target RNA substrate (red). Hammerhead ribozymes cut after a NUH consensus. (****B****) The hairpin ribozyme (black) and its target RNA substrate (red). Hairpin ribozymes cut following a NGUC consensus. There are 2 catalytic domains (****III****) that associate in a tertiary structure for hairpin ribozyme activity.*

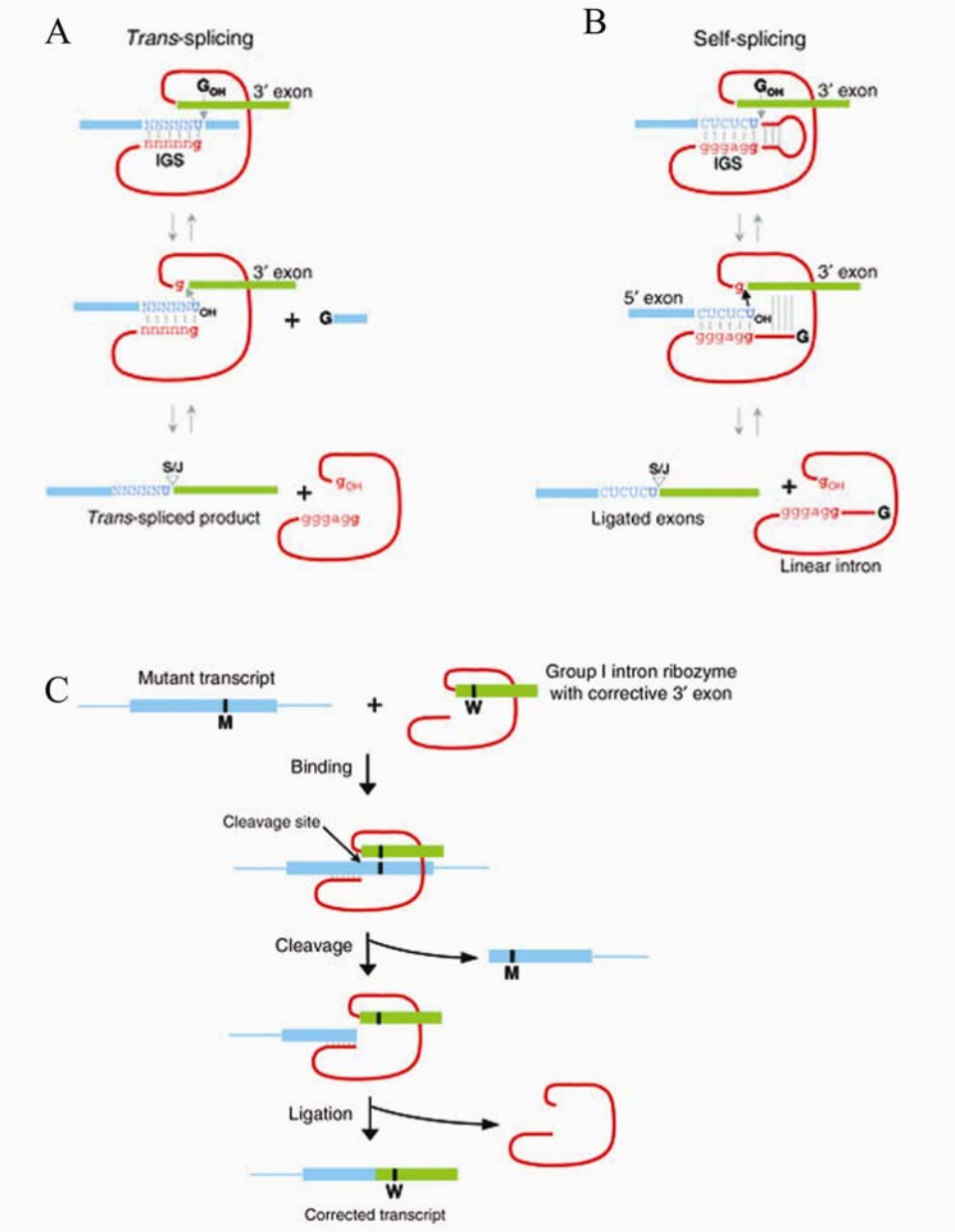

Figure 15 Self-splicing, trans-splicing and trans-splicing repair by the *Tetrahymena thermophila* group I intron ribozyme.

(A) *The trans-splicing reaction is similar to the natural self-splicing reaction* ***(B)*** *except that the target RNA is not covalently attached to the ribozyme. Exon sequences (blue and green) are shown as boxes and capital letters. Intron sequences (red) are shown as solid*

lines with lowercase letters. The targeted uridine residue is shown as a bold letter and the splicing junction (S/J) is indicated. ***(C)*** *Schematic representation of RNA repair using a trans-splicing ribozyme. A Group I ribozyme delivers corrective sequences (3′ exon) to a mutant transcript. The ribozyme binds upstream of the mutation through base-pairing. Once bound, the ribozyme cleaves the target RNA, releases the downstream cleavage product, and splices the 3'exon sequence onto the upstream cleavage product. M, mutant sequence; W, wild-type sequence corresponding to the mutated region. (Illustration adapted from (Long et al., 2003))*

1.2.3.3. Ribozyme design and applications

Expression systems and stability

It is essential in order to down-regulate the expression of a defective gene that the ribozyme be expressed continuously at high levels. There exists several RNA polymerase II (Pol II) expression systems, but these promoters may not be suitable for efficient ribozyme expression for several reasons. Pol II transcripts are processed for nuclear export and translation, such modifications could alter ribozyme folding and may also lead to translation of toxic peptides (Samarsky et al., 2000). To avoid RNA processing and the addition of extra flanking sequences, like the poly A tail for example, Pol III promoter systems have been employed. Pol III promoters are essentially responsible for the synthesis of small RNAs like tRNAs and snRNAs (small nucleolar RNAs) and can be effectively utilized to express functional ribozymes (Bertrand et al., 1997; Good et al., 1997; Samarsky et al., 2000). Pol III initiates transcription at a predetermined location (e.g. U6, H1), sometimes within the promoter sequence (e.g. tRNA promoters), and terminates transcription following a sequence of 5 thymidines. It is thus possible to incorporate stem loop structures flanking the ribozyme to confer additional stability and resistance to nucleases (Bertrand et al., 1997; Good et al., 1997).

Co-localization

A factor essential for maximum ribozyme efficacy is co-localization with the target RNA (Arndt and Rank, 1997; Bertrand et al., 1997; Lee et al., 1999; Lee et al., 2001; Michienzi et al., 2000; Samarsky et al., 1999; Sullenger and Cech, 1993). It is important to have the ribozyme accumulate in the same cellular compartment as its target whether it be the nucleus, the cytoplasm or the nucleolus. It is now easy to target such compartments by using the appropriate promoter system. For example, U3 promoters have allowed successful targeting in the nucleolus, a modified tRNAmet or U6 promoters to the nucleus and unmodified tRNA or certain Pol II snRNA and RSV promoters are best for cytoplasmic accumulation of ribozymes (Bertrand et al., 1997; Good et al., 1997). It is even possible to fuse localization sequences normally found in 3'UTR of mRNAs to the ribozyme transcript in order to direct it to a specific tissue *in vivo* (Lee et al., 2001).

Target selection

Another critical parameter in designing an active ribozyme is appropriate target selection. As with AS-RNAs and AS-ODNs, finding an accessible annealing site on the target RNA is the key to successful gene silencing. Avoiding long stretches of C-G repeats that form stem loops or regions known to recruit RNA binding proteins is a good start point. Several methods exist to identify accessible target sites since computer-assisted models cannot reliably predict RNA-protein interactions and RNA folding *in vivo* (Rossi, 1999). Effective methods employ the use of short AS-ODNs to measure RNAse H activity in cell extracts for each ribozyme target site tested (Ho et al., 1998; Mir et al., 2001; Scherr et al., 2000a; Scherr et al., 2000b). Basically, when a site is accessible for DNA-RNA pairing, the RNA component is rapidly cut by endogenous RNAse-H activity thus revealing a suitable ribozyme target site.

Applications

Since their discovery, ribozymes have been used mostly as tools to study gene function and target validation. But as our knowledge of their function increases, they are now being used in much more complex applications such the treatment of cancer, of genetic disorders and as antiviral agents. The review article located in Appendix 1 entitled 'Intracellular ribozyme applications' addresses current aspects of designing ribozymes for use as potential therapeutic agents (Castanotto et al., 2002).

1.2.4. RNA interference

Despite the hype surrounding RNA interference over the past 6 years, post-transcriptional gene silencing (PTGS) from dsRNA has been documented more than 15 years ago in plants and is not a new phenomena (Smith et al., 1990; van der Krol et al., 1988; van der Krol et al., 1990). It was then observed by several teams that expression of AS-RNAs or RNA from homologous genes completely abolished expression of genes with complementary sequences in plants, this was then termed co-suppression (Jorgensen, 1990). It was only in 1998 that this phenomena re-surfaced with a brand new name, RNA interference, in a publication by Fire and colleagues (Fire et al., 1998). In this work, they showed that a few molecules of dsRNA could abolish the expression of a complementary gene in *C. elegans*.

1.2.4.1. The siRNA pathway

Follow-up work on dsRNA-induced gene silencing in *Drosophila* embryo extracts revealed that long dsRNAs were processed into short 21-25 nt RNA duplexes with 2-nt 3'end overhangs and 5'-phosphate and 3' hydroxyl groups, termed short interfering RNAs (siRNAs) (Fig.16A) (Elbashir et al., 2001b; Zamore et al., 2000). While long dsRNAs are processed efficiently into siRNAs by various organisms, they however induce a non-specific interferon response in mammalian cells (Elbashir et al., 2001a; Jarvis et al., 1978; Pestka et al., 1987). The structure and characteristics of processed siRNAs are reminiscent of RNAse-III activity. It was later found that an evolutionary conserved RNAse-III-like

enzyme named Dicer was responsible for long dsRNA processing that is required for siRNA activity (Fig. 16B) (Bernstein et al., 2001; Ketting et al., 2001; Knight and Bass, 2001).

The way RNAi works is that long dsRNAs or small hairpin RNAs (shRNAs) of 19-25 nt in length are recognised by Dicer in the cytoplasm. Although some evidence points that there may be a nuclear component to RNAi that regulates chromatin remodelling (Volpe et al., 2002), reports have generally shown that both Dicer and RNA-induced silencing compex (RISC) activity are restricted to the cytoplasm (Dudley and Goldstein, 2003; Kawasaki and Taira, 2003; Paul et al., 2002; Zeng and Cullen, 2002). Dicer will then cleave in an ATP-dependent manner the long dsRNAs in precisely sized fragments (Dykxhoorn et al., 2003). If shRNAs are processed by Dicer, their stem loop will be cleaved to reveal the characteristic a 2-nt-3' overhang (Paddison et al., 2002). The resulting siRNAs then bound to Dicer will then pass through a siRNA-protein complex (siRNP) comprised of a helicase that will unwind and separate each homologous strand and direct them to RISC, the catalytic complex responsible for target RNA cleavage (Schwarz et al., 2003). Although Dicer associates with RISC, it does not participate in catalytic activity for target mRNA cleavage. Each RISC contains only one of the two strands of the siRNA, but both strands of the duplex may successfully induce RNAi if an appropriate target is available (Martinez et al., 2002). There is however recent evidence indicating that only the strand which is less tightly paired to its complement at the 5' end is incorporated into RISC, while the other strand is degraded (Schwarz et al., 2003). Interestingly, while artificial approaches for espressing or introducing siRNA in cells work well to induce RNAi, no naturally occurring siRNAs have yet been found in mammals (Dykxhoorn et al., 2003).

1.2.4.2. The miRNA pathway

Like siRNAs, micro RNAs (miRNA), or small temporal RNAs (stRNAs) named after RNAs that are temporally regulated, are capable of PTGS through translational repression

(Grishok et al., 2001; Hutvagner and Zamore, 2002; Lagos-Quintana et al., 2001; Lau et al., 2001; Zeng et al., 2003). MiRNAs are produced from Dicer-processing of shRNAs having imperfect sequence homology to the opposite strand of the duplex (Fig. 16C). The miRNA pathway shares many components of the siRNA pathway but differs in two main aspects (Zeng et al., 2003). For one, only the Dicer-processed antisense strand of the miRNA accumulates in the cell and is incorporated into the miRNA-protein complex (miRNP) (McManus and Sharp, 2002; Zamore, 2002). Secondly, PTGS is achieved by miRNAs through binding with partial sequence complementarity to their target RNA. It is this detail that can skew the response between target cleavage or translational repression (Zeng et al., 2003). Unlike siRNAs, miRNAs have been found in worms, flies and humans. (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) Most extensively studied are lin-4 (lineage-abnormal-4) and let (lethal-7) RNAs that control timing and sequence of postembryotic development in *C. elegans* (Ambros, 2001; Ambros, 2003; Ha et al., 1996; Lee and Ambros, 2001). They act as protein expression regulators by modulating transcription of their homologous mRNAs at precise moments in development (Pasquinelli and Ruvkun, 2002). Their discovery has opened our eyes to a world of tiny non coding RNAs that act as molecular switches to fine-tune gene expression (Storz, 2002).

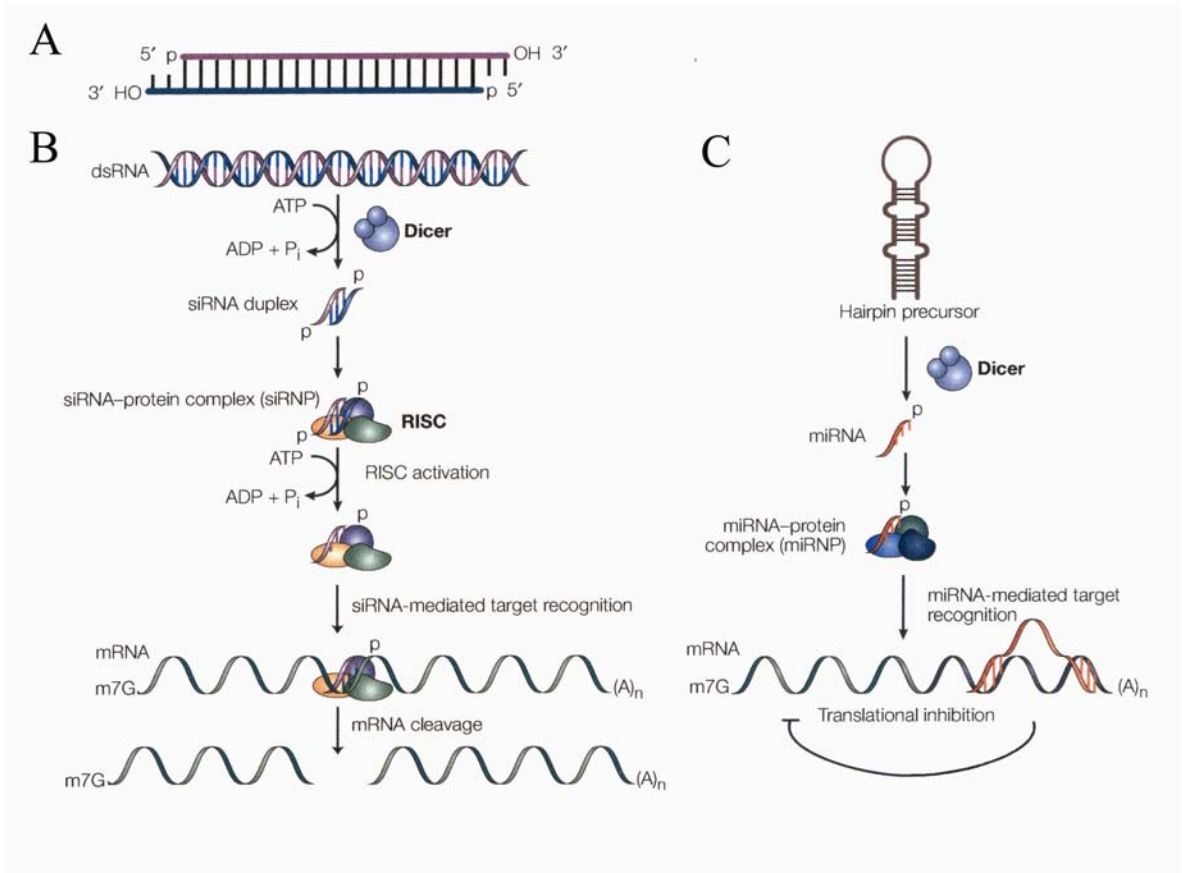

Figure 16 RNAi post-transcriptional gene silencing mechanisms

(A) *Short interfering RNAs (siRNAs) are generally between 19 and 27 nt in length with the characteristic 2-nt unpaired overhangs and 5'-phosphate and 3' hydroxyl groups.* ***(B)*** *The siRNA pathway to RNA interference. Long dsRNAs are processed by the RNAse-III-like enzyme Dicer into siRNAs. Processed siRNAs are then incorporated into the RNA-induced silencing complex (RISC) after being unwound and separated in the siRNP (siRNA-protein complex). Protein components of RISC will use a single strand of the previous siRNA duplex to home-in on an available target RNA with a complementary sequence. The target RNA will then be cut at the centre of the newly formed duplex between target RNA and the small antisense RNA.* ***(C)*** *The micro RNA (miRNA) pathway. Long, imperfect hairpin structures are also processed by Dicer to form single-strand miRNAs that are incorporated in the miRNA-protein complex (miRNP). These miRNAs then pair with partial complementarity to their target mRNAs leading to translational repression. (Illustration from (Dykxhoorn et al., 2003))*

1.2.4.3. RNAi design and expression systems

SiRNAs are expensive to synthesize, are rapidly degraded once transfected into cells, their silencing effects are transient in mammalian cells and not all siRNAs have the same cleavage efficiency (Hannon, 2002). This is why expression systems have been developed to stably express siRNAs or shRNAs and several factors need to be taken into account for proper siRNA or shRNA design (Elbashir et al., 2002; Lee et al., 2002; Paul et al., 2002; Xia et al., 2002). The 2 main promoter systems utilised today are: U6 and H1 promoters, both of which rely on Pol III for transcription and have given equally satisfactory results for producing siRNAs and shRNAs (Dykxhoorn et al., 2003; Tuschl, 2002). After deciding on a promoter system, one must next choose between producing a shRNA from a single promoter (Fig. 17A) or producing siRNAs that associate in *trans* from transcription products of tandem promoters (Fig. 17B). While shRNAs expressed from Pol III promoters have the disadvantage of requiring processing into siRNAs by Dicer, certain individuals have reported that fewer molecules of shRNAs than siRNAs are required to achieve RNAi (Hutvagner and Zamore, 2002; Paddison et al., 2002; Tuschl, 2002). This observation suggests that Dicer binding may facilitate entry of the processed shRNA into RISC (Dykxhoorn et al., 2003; Hutvagner and Zamore, 2002).

For other studies, it may be interesting to silence gene expression at the protein level. To this effect, one may consider using miRNAs that can be produced from inducible Pol II promoters expressing shRNAs with imperfect hairpin structures (Kennerdell and Carthew, 2000). An important consideration is that Pol II promoters incorporate regulatory sequences into the transcribed RNA that render them inadequate to form functional siRNAs, but work well as miRNAs (Fig.17C). The main drawback to using such a system is that these lengthier shRNAs (> 30 bp) may trigger an interferon response in mammalian cells and result in the non-specific degradation of RNA (Saunders and Barber, 2003; Scadden and Smith, 2001).

Another important part of designing a proper system for silencing specific genes through RNAi is the identification of accessible target sites. As for AS-RNAs and ribozymes, there is a correlation between RNAse-H accessible target sites and RNAi efficiency (Vickers et al., 2003). Therefore initial screening of the target RNA for good cleavage sites using AS-ODNs as described in the previous sections may help save a considerable amount of time.

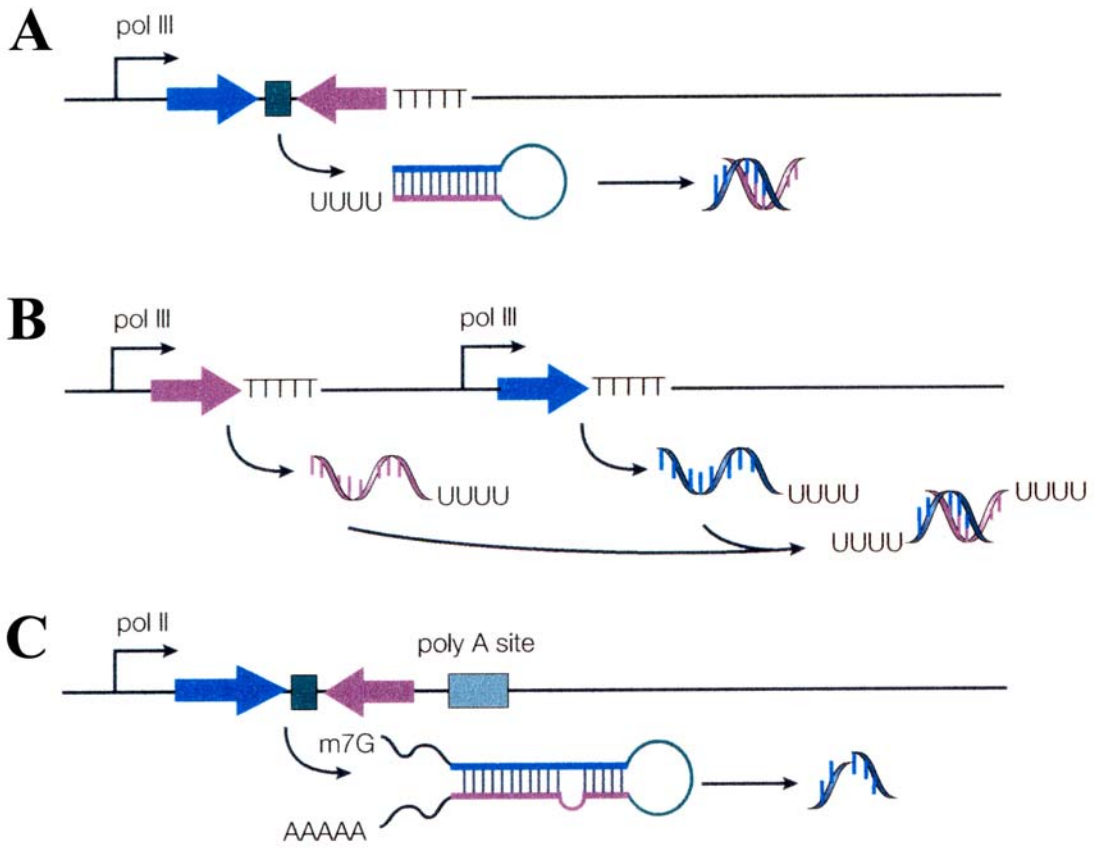

Figure 17 RNAi expression systems

(A) *A single Pol III promoter expresses a short hairpin RNA (shRNA) that will anneal in cis to form a functional siRNA.* ***(B)*** *Dual Pol III promoters drive the expression of individual small sense and antisense RNAs. These small RNA will anneal in trans to form functional siRNAs.* ***(C)*** *Pol II promoters are excellent tools for expressing imperfect hairpin RNAs. These will subsequently be processed by Dicer into mature miRNAs that can repress*

translation of the complementary mRNA in non-mamalian cells. (Illustration adapted from (Dykxhoorn et al., 2003)).

1.2.5. Delivery of small RNAs to cells

The major limitation in applying any of the previously discussed technologies is delivery. While it is possible *in vitro* to attain complete target destruction because all the cells express the desired therapeutic molecules at high levels, delivering these molecules for *in vivo* use is a completely different challenge. For short term target validation, the use of synthetic delivery systems is the cheapest, fastest and easiest way to go. However, in long term studies and for *in vivo* use, viral vectors offer the best results.

1.2.5.1. Synthetic gene delivery

This term regroups all non-viral methods to administer DNA or RNA into a cell including cationic lipids, polymers, electroporation and direct injection of DNA or RNA (Kootstra and Verma, 2003). These systems generally have low delivery efficiency, especially in primary culture cells, and provide only transient expression of transgenes. A novel system developed by Amaxa, combines both polymer/lipid reagents and electroporation. Although very expensive, this system is currently the most effective for delivering DNA and RNA to both cell lines and primary culture cells (Lenz et al., 2003).

1.2.5.2. Viral vectors

These are based on replication-competent viruses that have been genetically modified rendering them replication defective and less immunogenic through the deletion of accessory proteins (Kootstra and Verma, 2003). Although several viral vectors are now commercially available, the four that are most commonly employed are: adenoviruses (Ads), adeno-associated viruses (AAVs), the oncoretroviral murine leukemia virus (MLV)

and human immunodeficiency virus (HIV) -based lentiviruses. These viral vectors can be categorized into two subgroups, integrating (HIV and MLV) and non-integrating viruses (Ads and AAVs) (see table 6 for more details) (Kootstra and Verma, 2003). In order to develop an effective therapy for myotonic dystrophy, the ideal viral vector must be able to transduce differentiated myofibers and sustain transgene expression for prolonged periods.

The Adenovirus (Ad)

Ads enter cells through binding to the ubiquitously expressed receptor CAR (coxsackievirus and adenovirus receptor) (Bergelson et al., 1997). Ads can infect both dividing and non-dividing cells, but the viral DNA exists in episomal form which can be diluted from rapidly dividing cells. Although first and second generation Ads raised many safety concerns about the emergence of replication competent viral particles through recombination with complemented sequences, gutless Ads show much promise in gene therapy (Kootstra and Verma, 2003). These Ads are deprived of nearly all viral genes and necessitate a helper virus providing the required viral proteins for replication and encapsidation. Risks of producing replication competent particles are minimized because of the utilization of producer cells expressing Cre recombinase that removes from the helper virus the packaging signal flanked by loxP sites. Furthermore, absence of viral genes in the gutless Ad allows a DNA cargo of up to 29 kb to be inserted into the virus. A minimal cargo of 27 kb is essential for packaging (Kootstra and Verma, 2003).

The Adeno-associated viruses (AAV)

AAVs are known to efficiently transduce adult muscle. However not all AAV serotypes infect with the same efficiency. AAV6 is far better than AAV1 which is a 100-fold more efficient than AAV2 (Hauck and Xiao, 2003). The main disadvantage of using AAVs are the difficulty in obtaining high viral titers necessary to infect muscle *in vivo* and the fact that they don't efficiently integrate to the target cell genome. This feature may prove problematic in situations where proliferating myoblasts need to be transduced before being

allowed to differentiate into myotubes for studies *in vitro*. Also, the limited DNA payload capacity of this vector (< 4.7 kb) severely restricts the space available for the transgene, the selection marker and their respective promoters needed to identify positive clones.

The lentivirus

A very promising viral vector for muscular dystrophy is the HIV-based lentivirus. This retroviral vector can be produced at high titers, can infect both dividing and non-dividing cells and can be pseudotyped with the glycoprotein of the experimenter's choice (Kootstra and Verma, 2003). Additionally, as opposed to Ads, these viruses do not induce a CTL (cytotoxic T-cell lymphocyte) response. To address safety concerns, genetic engineering has produced a lentiviral vector system that that is self-inactivating (SIN) (Yu et al., 1986). These vectors have a large deletion in the 3' U3 region that contains the viral promoter and enhancer. This deletion is copied over to the 5' region during reverse transcription which in turn completely abolishes LTR-driven transcription in transduced cells (Kootstra and Verma, 2003). Furthermore, lentiviral vector systems can contain between 7.5 to 9 kb of cargo DNA, which is largely sufficient for cloning transgenes, selection markers and the promoters needed for their expression. This is an important feature, especially when planning to use lengthy inducible or tissue-specific promoters. Finally, lentiviral vectors have been shown to successfully transduce and stably express transgenes or siRNAs in muscle cells making them the delivery system of choice for muscle-oriented gene therapy (Kafri et al., 1997; MacKenzie et al., 2002; Rubinson et al., 2003).

Table 6 Properties of viral vector systems

Features	AAV	Adenoviral		Retroviral	
		Adeno (1st & 2nd gen.)	Adeno (Gutless)	Lenti	Onco
Viral genome	ssDNA	dsDNA	dsDNA	RNA	RNA
Max. transgene size (kb)	< 4.7	~ 8.3†	27-29	~ 7.5 - 9†	~ 7.5†
Integration	No (Yes?) *	No	No	Yes	Yes
Non-dividing cell transduction	Yes	Yes	Yes	Yes	No
Duration of expression	Long	Short	Long	Long	Long
Induction of CTL response	No	Yes	Yes	No	No
Pre-existing immunity	Yes	Yes	Yes	No‡	No
Safety concerns	Insertional mutagenesis?	Inflammation cytotoxicity		Insertional mutagenesis	

**It is still unclear whether recombinant AAVs are capable of stable integration.*

† DNA payload size depends on the presence of accessory proteins

‡Except for HIV patients

Part III: Research hypothesis and objectives

1.3. Hypothesis

Myotonic dystrophy is a crippling and devastating neuromuscular disease. To this date there is no cure, however several drugs exist that relieve certain symptoms of the disease, but no drug is without risks and side-effects. Furthermore, since a vast number of systems and organs are involved in myotonic dystrophy several drugs must be taken simultaneously to help relieve the symptoms, at the cost of multiplying the harmful side-effects. Considering this, a single, more generalized approach would be far more appropriate to treat myotonic dystrophy than drug-based approaches than only partially relieve certain symptoms of the disease, and do nothing to restore muscle strength.

Over recent years, it has become clear that the molecular mechanism responsible for the vast majority of cellular dysfunctions in myotonic dystrophy is the nuclear retention of DMPK mRNA with large CUG repeats. Our working hypothesis is that a gene therapy approach that specifically targets and destroys mutant transcripts would ultimately rescue DM1 myoblasts from several of the disease features such as: splicing aberrations, insulin resistance and defects in myoblast differentiation and fusion.

1.4. Objectives

Although certain attempts have been made to treat myotonic dystrophy by gene therapy *in vitro*, none of these methods were shown to be successful in restoring a normal phenotype in affected cells (Galderisi et al., 1996; Melone et al., 1998; Phylactou et al., 1998a). Our work has focussed on four key points to provide tools in order to develop a successful gene therapy approach for eventual treatment of myotonic dystrophy patients:

1) **Development of a human myotonic dystrophy type I (DM1) primary cell culture model for *in vitro* studies.**

2) **Development of a proper diagnosis tool for measuring independently normal and mutant DMPK mRNA expression levels.**

3) **Evaluation of RNA-based approaches capable of reducing mutant DMPK mRNA expression and restoring normal phenotype features in DM1 myoblasts.**

4) **Identification and characterisation of a novel CUG-binding protein implicated in DM1 pathogenesis that could serve as a new therapeutic target or molecule.**

CHAPTER II

Increased stability of mutant DMPK mRNA in congenital myotonic dystrophy

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Running title: Increased mutant DMPK mRNA stability in congenital myotonic dystrophy

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2.1. Résumé

La dystrophie myotonique de type 1 (DM1) est une maladie neuromusculaire causée par une expansion du triplet CTG dans la région 3' non-codante (3'UTR) du gène de la dystrophica myotonica protéine kinase (DMPK). Les patients atteints de la forme congénitale de la maladie présentent des symptômes sévères dès la naissance. Malgré que les formes courantes et congénitales de la maladie sont toutes les deux causées par la même mutation, certains caractéristiques moléculaires permettent de différencier chacune de ses formes. Dans ce travail, nous démontrons que des myoblastes isolés de deux fœtus atteints de la forme congénitale de la maladie expriment 61% et 63% plus de ARNm provenant du gène ayant la mutation. Ces niveaux élevés d'ARNm mutants résultent non pas d'un taux de transcription plus élevé de l'allèle mutée tel qu'il a été postulé, mais bien d'une plus grande stabilité des messagers mutés comparés aux messagers normaux de DMPK. Cette augmentation de messagers mutés de DMPK n'a pas été observée dans des myoblastes provenant de fœtus ayant la forme courante de la maladie. Ces résultats suggèrent qu'il existe un mécanisme qui stabilise les ARNm mutés de DMPK dans les myoblastes de forme congénitale.

2.2. Abstract

Myotonic dystrophy type 1 (DM1) is an autosomal dominant neuromuscular disorder caused by a CTG expansion in the 3'untranslated region of the myotonic dystrophy protein kinase (*DMPK)* gene. Patients with the congenital (CDM1) form of the disease experience severe symptoms at birth as opposed to the degenerative, late onset phenotype seen in adult cases (DM1). While both forms of the disease share the characteristic trinucleotide amplification, molecular mechanisms leading to the severe CDM1 form are poorly understood. Here, we report that myoblasts isolated from congenitally affected DM1 fetuses express 60% more mutant DMPK mRNA compared to the normal transcripts. This elevation was not detected in DM1 myoblasts that were not diagnosed with the congenital form of the disease. Elevated mutant DMPK mRNA levels result from increased mRNA stability in CDM1 cells and not from increased transcription of the *DMPK* gene. These data suggest post-transcriptional stabilisation of the mutant DMPK mRNA in CDM1 myoblasts that could participate in the aggravated phenotype.

Key words: Congenital myotonic dystrophy, DMPK, nucleus.

2.3. Introduction

Myotonic dystrophy type 1 or DM1 is a autosomal dominant neuromuscular disorder caused by a CTG expansion in the 3'untranslated region of the myotonic dystrophy protein kinase (*DMPK)* gene (1,2). Mainly, two forms of DM1 disease exist with distinctive clinical symptoms: adult and congenital. The degenerative, late onset adult form (DM1)[1] is characterized by myotonia, muscle weakness and wasting, cataracts and insulin resistance (3). The more severe congenital (CDM1)[1] form present at birth is associated with hypotonia, mental retardation and delayed muscle maturation. Globally, there is a correlation between the severity of symptoms and the length of the CTG amplification. In fact, unaffected individuals have fewer than 38 CTG repeats, whereas DM1 patients have more than 100 CTG repeats and patients affected by the severe congenital form have extremely large CTG expansion (>1500 CTG). The physiopathological mechanisms leading to DM1 are still not thoroughly understood. Several hypotheses have been proposed to explain how the CTG amplification causes the complex, multisystem DM1 phenotype. The explanation that has gained the most support attributes the toxic effects directly to the mutant DMPK transcripts containing the large CUG repeat expansions. However, little is known about the additional molecular mechanisms responsible for generating the congenital form with the characteristic impaired skeletal muscle development (4). The mutation causing both forms of the disease, DM1 and CDM1, is the same. For the moment, the only reliable molecular feature to distinguish both forms of the disease is that CpGs proximal to the mutation in the *DMPK* gene are aberrantly methylated in congenital cases (5,6). Certain CpG islands of the *DMPK* gene are located within the binding region of the zinc-finger protein CTCF (6). Methylation of the CTCF binding site results in loss of insulator activity (6,7) and could interfere with *DMPK* gene expression in CDM1.

The expression of mutant DMPK transcripts plays a central role in the development of myotonic dystrophy. It has been demonstrated that the mutant DMPK mRNA with more than 150 CUG repeats are fully retained in the cell nucleus and form foci visible by *in situ*

hybridization (8-11). The nuclear retention of the mutant DMPK transcripts harbouring large CUG repeats create a gain of function that disrupts processing of heterologous RNAs in a *trans* dominant manner. The mutant transcripts interact with various RNA binding proteins such as CUG-BP and muscleblind family members causing numerous cellular dysfunctions and splicing abnormalities (10-18).

Several reports have determined the steady-state levels of the mutant and normal DMPK mRNA but no consensus has been attained on whether or not mutant transcript expression and polyadenylation are altered in DM1 (9,19-24). A possible cause leading to the multitude of contradicting reports is an improper and incomplete extraction of DMPK mutant transcripts. In fact, the strong association of mutant DMPK transcripts with nuclear matrix proteins is thought to prevent their export from the nucleus (9). Many techniques have been used in order to extract the mutant transcripts from the nuclear aggregates in DM1 cells, but no effective and reliable method of extraction has been established. Quantitative studies using Northern blot and RT-PCR can be biased if extractions of the cytoplasmic and nuclear-retained transcripts are not achieved completely. Mutant transcripts can thus be under-represented in the total DMPK mRNA pool.

The aim of the present study was to determine whether the expression of the mutant DMPK transcripts are differently regulated in DM1 and CDM1 myoblasts isolated from muscles with various CTG expansions. In order to shed light on DMPK expression levels in DM1, we have set-up a method that fully and reliably extracts the RNA containing CUG repeats. A proteinase K digestion of the cellular preparations prior to RNA extraction was used in order to obtain complete degradation of cellular proteins thus facilitating RNA isolation. We show that the steady-state levels of both mutant and normal DMPK transcripts are similar in DM1 myoblasts. In contrast, the mutant DMPK transcripts are significantly increased in CDM1 myoblasts. Higher expression of mutant DMPK mRNA does not result from transcription discrepancies between the normal and the mutant alleles but from increased mutant DMPK mRNA stability in congenital myoblasts. We hypothesize that

these results lend support for a mechanism wherein mutant transcripts are stabilized through high-affinity binding to double-stranded RNA binding proteins in the nucleus of CDM1 cells that may be accountable for the severe symptoms typical of the congenital form of the disease.

2.4. Materials and methods

Primary human myoblast cultures

Control myoblasts were obtained from the skeletal muscle of a normal aborted fetus. DM-750, DM-1200 and DM-3200 myoblasts were obtained from the skeletal muscles of aborted DM1 fetuses with approximately 750, 1200 and 3200 CTG repeats, as verified by Southern blot. Because these myoblasts were obtained from aborted fetuses, it was not determined if they had DM1 or CDM1. DM-3700 myoblasts were obtained from a deceased CDM1 neonate. This neonate had 3700 CTG repeats and presented all the typical clinical features of the congenital form of the disease with various abnormalities in feet, in pulmonary growth and presented muscular hypotrophy. All biopsies were obtained in accordance with the Laval University Medical Research Centre ethical committees.

Myoblasts were grown in MCDB-120 supplemented with 15% heat-inactivated fetal bovine serum, 10 µg/ml insulin, 0.5 mg/ml BSA, 10ng/ml human EGF, 50 µg/ml streptomycin and 50 µg/ml penicillin. For myoblast differentiation, growth medium was removed at 90% culture confluence and replaced by DMEM supplemented with 0.5% heat-inactivated fetal bovine serum, 10 µg/ml insulin, 0.5 mg/ml BSA, 50 µg/ml streptomycin and 50 µg/ml penicillin.

RNA isolation

Three different techniques were used to isolate total RNA. Total RNA extraction was performed either using the Trizol® reagent (Life Technologies) according to the manufacturer's specification or using 4M guanidium thiocyanate buffer followed by a CsCl purification as described elsewhere (9). The third protocol was developed using a proteinase K digestion in the first step of isolation. Briefly, three ml of fresh lysis buffer

was added to a 60mm dish. The lysis buffer contained the following: 500 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1.5 mM $MgCl_2$, 10 mM EDTA, 2% SDS and 0.50 mg/ ml of proteinase K. The cells were harvested after detaching from the dishes and incubated at 45^{o}C for 45 minutes in a shaking water bath. Then, nucleic acids from the proteinase K lysates were then precipitated by adding 300 µl of NaOAc 3M and 8.25 ml of pure EtOH. Tubes were spun for 20 minutes at 4^{o}C and pellets were resuspended in 100µl water and 1 ml Trizol reagent was added. Total RNA extraction was then carried out according to the manufacturer's specifications. Alternatively, PolyA(+) mRNA were also directly isolated after the first step of proteinase K digestion. Messenger RNA were extracted from the proteinase K lysates using twenty-five mg of oligo (dT)-cellulose Type 7 (Amersham Pharmacia Biotech inc., Piscatawa, N.J.) and isolated as described previously (25).

Northern blot

Ten micrograms of total RNA or 500 ng of poly $(A)^+$ mRNA were loaded on a 1.2% agarose gel with 2.2M formaldehyde and 1 X MOPS. Samples were separated by electrophoresis and then transferred to a Biodyne B nylon membrane (Pall Corp., Ann Arbor, MI) using a microfiltration apparatus (Pharmacia). Hybridization conditions were previously described (25). The labelled DMPK probe used was prepared by random priming of 50 ng of the BglII-SacI fragment of the DMPK cDNA with ^{32}P-dCTP. The cyclophilin probe was prepared by random priming of a 700 bp PCR product amplified from cyclophilin cDNA.

Quantifications

Densitometry analysis was done on the original autoradiograms with an AlphaImager imaging system and software (Innotech Corp.) by quantifying each band independently. For figures 1,3 and 4 the relative intensity of each band was determined as follows: a standard value (V) was determined independently in each panel by dividing normal (N_1) transcript expression in the first lane over its corresponding cyclophilin (C_1) expression. Levels of

both mutant (Mut_n) and normal (N_n) transcripts in all subsequent lanes were then divided by their corresponding cyclophilin expression followed by this standard value to determine relative intensity (e.g. $Mut_n/C_n/V$ or $N_n/C_n/V$). Division of normal transcript expression in lane 1 over its corresponding cyclophilin expression over the standard value gives a relative intensity of 1 ($N_1/C_1/V = 1$). In figure 5, the standard value for each panel was determined by dividing expression of the normal transcript at T=1 over its corresponding cyclophilin expression. This was necessary since DMPK is weakly expressed in proliferating myoblasts. In figure 7, a standard value was determined independently for normal and mutant transcripts thereby establishing both normal and mutant transcript expression at T=0 as 1.

Southern blot

Myoblasts were harvested and digested with proteinase K followed by nucleic acid precipitation as described earlier. Pellets were resuspended in water containing 100 µg/ml RNAse A. Fifteen µg genomic DNA was used in every digestion with appropriate restriction enzymes and samples were purified by phenol-chloroform extraction prior to gel loading. DNA was resolved on a 0.8% agarose gel, blotted onto a Biodyne B nylon membrane and hybridized with the labelled DMPK probe according to standard procedures. The genomic DMPK probe was generated by PCR using the following primers: Forward: gagctccaggaagccctgga; Reverse: cccttcgagccccgttcgcc. Labelling was done with ^{32}P-dCTP using the reverse primer.

Slot Blots

Three µl of NaOH 5 M was added to 135 ng of CTG-80 probe (250 bp), 790 ng of the genomic DMPK probe used for the Southern blot (1580 bp), 5 µg of pBluescript KS+ vector and to 350 ng of cyclophilin probe (700 bp), samples were then adjusted to 50 µl with water. Samples were then boiled for 5 minutes and cooled on ice. Fifty µl of 4M

ammonium acetate was added and samples were applied to a nylon filter inserted in a slot-blot apparatus. Slots were washed twice with 2 X SSC and the membrane was UV crosslinked for 3 minutes and cut accordingly.

Nuclear Run-on assays:

Isolation of nuclei and synthesis of radiolabeled nascent RNA were done as described here (26) with the following modifications: transcriptions were carried out for 10 minutes in presence of 200 μCi ^{32}P-ATP per sample to maintain similar specific activity between normal and mutant DMPK transcripts. Total labelled RNA was extracted from the samples using the method described earlier. Hybridization was done overnight in Northern blot hybridization buffer containing 200 μg/ml of *E. coli* tRNA. All DNA probes were blotted in molar excess of complementary endogenously transcribed RNA. Mutant but not normal DMPK mRNA binds strongly and specifically to the $(CTG)_{80}$ probe. All assays were done in duplicate and carried out simultaneously with or without 400 μg/ml α-amanitin which successfully blocked transcription in all samples (Data not shown). DMPK mRNA expression normalizations were done according to cyclophilin transcription levels in each sample (Data not shown).

2.5. Results

Normal and mutant DMPK mRNA levels are similar in DM1 myoblasts

The steady-state levels of DMPK mRNA were measured in human myoblast cultures derived from normal and DM1 muscles with 750 and 1200 repeats (Fig. 1A). We tested three commonly used lysis buffers: guanidium thiocyanate, Trizol and proteinase K, and compared their efficiency to extract mutant DMPK mRNA with large CUG repeats (see experimental procedures). For each method, total RNA was isolated from myoblast cultures differentiated for 3 days and relative expression of normal and mutant DMPK mRNA was quantified by Northern blot.

While all methods yield complete extraction of the normal transcripts in DM1 myoblasts, mutant transcript extraction efficiency is variable between methods (Fig. 1A). Cyclophilin normalization allows extraction efficiencies to be compared between techniques. The guanidium thiocyanate buffer and Trizol reagent do not fully extract DM transcripts with the large CUG expansions. Both these methods yield approximately 60-65% extraction efficiency as compared to the proteinase K method (Fig. 1B). Using the proteinase K method, we show that expression of normal and mutant DMPK transcripts are similar in DM1 muscle cells. These results also indicate that proteolytic digestion of samples is a requirement to fully achieve mutant mRNA extraction.

The DMPK locus in CDM1 myoblasts is aberrantly methylated

In order to compare DMPK steady-state levels between DM1 and CDM1 myoblasts, we needed to confirm which form of myotonic dystrophy was present in our myoblast cultures. DM-3700 myoblasts were isolated from a deceased neonate that had typical clinical features of the congenital form. However, the clinical phenotype for DM-750 and DM-

3200 myoblasts was unconfirmed because of the early age of the fetuses (20 weeks and 15 weeks respectively). The only reliable feature to diagnose the congenital form of the disease at the molecular level is to examine the proximal region of the CTG expansion that was shown to be aberrantly methylated in CDM1 (5,6)

Thus, we have determined the methylation status of the Normal, DM-750 and DM-3200 myoblasts. DNA prepared form the various cells was digested using methylation-sensitive Sac-I, Sac-II and Hpa-I restriction enzymes and a Southern blot was performed as previously described (5). Figure 2A shows a restriction map of the 1.8 Kb region of interest in the DMPK gene and the recognition sequence for the DMPK probe used here. Normal *DMPK* alleles generate a single 1.8 kb fragment following co-digestion by Sac-I and Hind-III (SI/H) and mutant alleles generate a band augmented by the corresponding length of the CTG expansion (Fig. 2B). While the upstream Sac-II site (360) is constitutively methylated in Normal and DM1 cells, the Sac-II site (1560) proximal to the repeat tract has been shown to be aberrantly methylated in CDM1 cells (5). Triple digestion with Sac-I, Sac-II and Hind-III (SI/SII/H) results in loss of the expansions in Normal and in DM1 myoblasts (DM-750) and generate a 1.6 kb fragment. On the other hand, in CDM1, the expansion is retained in the digested fragment and generate a fragment of (11.4 kb) in the cells with 3200 CTG repeats (DM-3200). Triple digestion with Sac-I, Hind-III and Hha-I (SI/H/Hha) results in 0.9 kb bands showing that the repeats are not contained in a fragment located between Sac-II sites, thus showing that Hha-I sites proximal to the repeats are unmethylated. These restriction assays permit to confirm that DM-3200 was affected by the congenital form since methylation occurs only in the mutant *DMPK* gene of CDM1 myoblasts.

CDM1 myoblasts express more mutant DMPK mRNA than DM1 myoblasts

We showed in figure 1 that the steady-state levels of normal and mutant DMPK transcripts are similar in DM1 myoblasts containing 750 and 1200 CTG repeats. Using the proteinase

K digestion followed by total RNA extraction, we examined DMPK mRNA expression in CDM1 myoblasts with 3200 and 3700 CTG repeats (Fig. 3A). Figure 3B shows that CDM1, DM-3200 and DM-3700 myoblasts express on average 61% and 63% more mutant than normal DMPK mRNA respectively. A similar result was also obtained from DM-3200 skeletal muscle tissue, confirming the increased in DMPK mutant transcripts in CDM1 (Fig. 3A). The complete extraction of mutant DMPK mRNA from CDM1 tissue using the proteinase K digestion displays the usefulness and efficiency of this method. Since our results were obtained from total RNA extractions, we were interested in examining whether polyadenylation was affected or not in DM1 and CDM1 myoblasts. Our results show that both the total RNA (T) and the polyadenylated (A+) mRNA fractions of DMPK transcripts display similar expression levels and no un-polyadenylated (A-) DMPK RNAs were present in the samples (Fig. 4A). Figure 4B shows RNA loading and purity on an agarose gel stained with ethidium bromide. As measured with the total RNA extraction, polyadenylated mRNA from congenital DM-3200 cells also showed an increase in the expression of the mutant DMPK transcripts by approximately 60% when compared to normal transcripts (Fig. 4C).

Transcription rates of normal and mutant *DMPK* alleles are the same in CDM1 myoblasts

In order to determine if the higher levels of mutant transcripts in CDM1 cells result from a specific increase in the transcription rate of the mutant *DMPK* allele, nuclear run-on assays were performed. A $(CTG)_{80}$ probe was used to detect specifically the mutant transcripts. Figure 5A depicts a Northern blot showing that this probe only binds to mutant transcripts. Normal DMPK mRNA have between 5 and 16 CUG repeats in the myoblasts assayed which is not sufficient to allow binding to the $(CTG)_{80}$ probe in our hybridization conditions. Normal DMPK transcripts were revealed using the DMPK probe used for the Southern blot, and a cyclophilin cDNA probe was used for normalization and transcription controls. All the blotted probes were in molar excess to the labelled RNA to insure depletion of transcribed RNA in each sample. Negative binding controls were insured by plasmid DNA on the slot blots. Nuclei from Normal, DM-750 and DM-3200 myoblasts

were prepared on ice and transcription was carried over for 10 minutes using ^{32}P-ATP. Radiolabeled ATP was used to insure that normal and mutant transcripts had a similar specific activity, since any other ribonucleotide would have resulted in greater labelling of the mutant transcripts. Total radiolabeled RNA was then extracted from the nuclei and hybridized (Fig. 5B). Dot blot analysis indicates that DM-750 and DM-3200 myoblasts display similar transcription levels for both normal and mutant transcripts. Since the transcription rate is not altered in CDM1 cells, this strongly suggests that the higher levels of mutant DMPK mRNA in the nucleus of CDM1 myoblasts may be caused by increased mutant DMPK mRNA stability.

Mutant DMPK mRNA is more stable in CDM1 myoblasts

To investigate the proposition of a higher mutant DMPK mRNA stability in CDM1 cells, RNA stability was assessed using actinomycin D. DM-750 and DM-3200 cultures were treated with 5 μg/ml of actinomycin D for 24 hours. No mortality was observed at the final time point assayed. RNA was extracted at different time points and DMPK expression was analysed by Northern blot (Fig. 6A). These experiments indicate that the DMPK mRNA transcribed from the healthy allele has a half-life of approximately 9.6 to 10.2 hours in all cells tested (Fig. 6B). While mutant transcripts in DM-750 cells agree to the same rule. In contrast, mutant transcripts in congenital DM-3200 cell show prolonged persistence in cells with a half-life of 18.6 hours.

To evaluate the effect of such an increased stability of the mutant DMPK transcripts in CDM1 muscle cells, we measured the steady-state levels of the DMPK mRNA during myoblast differentiation. Figure 7A shows that DMPK mRNA is very weakly expressed during myoblasts proliferation (T=0), but is rapidly induced upon serum deprivation. After 24h in differentiation medium, a 5-fold increment in DMPK mRNA levels was measured in Normal, DM1 and CDM1 cultures. The levels of mutant and normal transcripts were similar in DM1 cells at all times during differentiation in contrast to CDM1 myoblasts

(DM-3200) that showed 60% more mutant DMPK mRNA compared to the normal DMPK transcripts (Fig. 7B). While all cells show a reduction of approximately 25-35% in both normal (N) and mutant (Mut) steady-state DMPK levels at day 4, levels of mutant transcripts in DM-3200 myoblasts persist as they are only 18% below the level seen at day 1. The mutant mRNA accumulation measured during myoblast differentiation reflects the elevated stability of mutant transcripts measured previously. Since this process occurs mainly during differentiation, the debate regarding the existence of other gene products involved in stabilizing mutant transcripts thereby causing their accumulation during CDM1 myoblasts differentiation is still open.

2.6. Discussion and conclusions

We have highlighted in this report that a proper and efficient method to extract mutant DMPK mRNA that are firmly anchored to the nuclear matrix, is an absolute pre-requisite for studying DMPK expression ratios. We showed that a proteinase K digestion of DM1 and CDM1 samples prior to Trizol extraction is the most effective method tested to extract the mutant transcripts that are sequestered in the nucleus. Trizol and guanidium isothiocyanate methods did not prove to be effective in extracting nuclear RNA with large CUG expansions in human skeletal myoblasts. Incomplete mutant DMPK mRNA extraction combined with flawed experimental approaches can explain the multitude of controversial reports on DMPK mRNA expression in DM1. It has been reported in the literature that mature DMPK mRNA transcribed from the mutant allele in DM1 is either not expressed (21,27,28), reduced (9,20,22-24), elevated (19) or unchanged (29).

By shedding a critical look at these reports we must set apart studies done with DM1 and CDM1 cells. Firstly, we must rule-out studies that did not detect mutant DMPK mRNA because of ineffective extraction methods (21,27,28). It is now widely accepted in the DM field that mutant DMPK mRNA is expressed in all DM1 cells. In an early publication, Fu et al reported reduced levels of mutant DMPK mRNA in adult DM1 myoblasts (20). They tried to detect differential expression of mutant and normal transcripts using primers flanking the repeats sequences. It was later shown that PCR cannot be reliably performed to amplify large CTG expansion (19). A few years later, results by Wang (23) and Krahe (22) also showed reduced levels of mutant DMPK mRNA extracted from adult DM1 cells. Both these studies used a similar product to Trizol to extract the RNA. We demonstrated that Trizol was ineffective in completely extracting mutant DMPK RNA. Two other reports published simultaneously also looked into DMPK mRNA expression levels in fibroblasts isolated from DM1 adults (9,24). While in the first study (9) the authors showed a 30 to 70% reduction of mutant DMPK RNA levels in myoD-transduced fibroblasts, in the second paper the authors describe a reduction of mutant DMPK mRNA, but only in the poly (A)+

fraction (24). Discrepancies between these results and ours may be explained by the use of different mRNA extraction methods and the fact that they used myoD-transduced fibroblasts instead of the primary culture myoblasts we used in our study. Lastly, a publication by Bhagwati et al (29) shows that in adult DM1 myoblasts there is equal expression of both mutant and normal DMPK transcripts using carefully chosen primers for allele-specific PCR. These results are in perfect accordance to those we present in this report.

The most convincing work with CDM1 cells was performed by Sabourin and colleagues (19). They demonstrated that expression levels of total DMPK transcripts in various CDM1 tissues are increased using dot blots. They then showed that this elevation was caused by the accumulation of mutant DMPK RNA. They propose that this increase is a result of either increased stability or elevated levels of mutant allele transcription (19).

To resume current knowledge on DMPK expression based on published literature and our current results, we conclude that the normal and mutant alleles in DM1 tissue are expressed at the same level. In CDM1 tissue however, mutant DMPK transcripts are more abundant because of increased stability. It is now clear that correct RNA extraction procedures using proteinase K and usage of an unbiased detection technique such as Northern hybridization may avoid discordant results in the future. Also, complete digestion of cellular extracts by proteinase K may be essential in completely dislodging nuclear-retained mutant DMPK mRNA.

Little work has been done on CDM1, and until recently, it was virtually impossible to distinguish at a molecular level between DM1 and CDM1 cells. CDM1 is always associated with very large amplification (>1500 CTG). However, the length of the repeat tract alone does not suffice to predict a congenital phenotype since some DM1 patients also have large CTG amplifications similar those measured in congenital cases but do not

develop this severe form. Hypermethylation of the region that flank the CTG repeats was until now the only molecular feature that clearly established the congenital form (5). Two CTCF-binding sites were found in these aberrantly methylated regions (6). CTCF is a functional part of insulator elements capable of blocking promoter-enhancer interactions in vertebrates and methylation of the CTCF binding site results in the loss of insulator activity at the DM1 locus (7,30). Because this insulator is located between the DMPK gene and the Six5 enhancer, it has been postulated that it could interact with the DMPK promoter (6). The consequences of this methylation interference remain to be determined since transcription levels of normal and mutant DMPK alleles are similar.

The finding that there are elevated levels of mutant DMPK in the nucleus may be helpful in explaining the severity of the CDM1 phenotype. We have shown that the steady-state levels of mutant DMPK transcripts are increased by 60% in CDM1 muscle cells. Furthermore, we demonstrated that this increase is the direct consequence of elevated mutant transcript stability rather than higher transcription levels of the mutant allele. It is known that terminal myoblast differentiation can be inhibited by overexpressing short CTG repeat tracts (~200) expressed from a strong CMV promoter (31). Therefore accumulation of stable mutant DMPK mRNA with long CUG repeat tracts in the cell nucleus may be involved in the delay of muscle differentiation seen in CDM1 patients.

These two molecular particularities of CDM1 bring forward strong support that protein expression is differentially regulated in these cells. Until now, the major cellular and molecular defects seen in DM1 were though to relate to the nuclear retention of the mutant DMPK mRNA leading to the toxic gain-of-function mechanism. Evidence showing that the CTG/CUG expansions alone do not suffice to produce the DM1 or CDM1 phenotype is provided by work done on myotonic dystrophy type 2 (DM2)1 (32,33). DM2 is caused by a large and unstable CCTG expansion in intron 1 of the zinc finger protein 9 gene and shares most common clinical symptoms with DM1 such as the myotonia, cataracts, muscle weakness and wasting, (32-34). The main clinical difference between these two forms of

myotonic dystrophy resides in that DM2 patients have dominant symptoms in proximal muscle groups are as compared to distal muscle groups in DM1 (35). At a molecular level, RNA carrying the DM2 expansion are sequestered in the nucleus of DM2 cells and form foci that recruit the same RNA binding proteins as in DM1 (10). Just as DM1 and DM2 are very similar in all major aspects of the disease, there are no documented cases of congenital DM2 (35). Also, the length of the CCTG tract in DM2 patients often exceed 10 000 repeats without causing the severe phenotype observed in CDM1 (36).

Our evidence supports the hypothesis that there may be a temporal component in producing the CDM1 phenotype. It is indisputable that certain critical molecular events occur during fetal development that lead to the particular CDM1 phenotype at birth. Because the mutation causing DM1 and CDM1 is the same and that foci in cells with either form of DM seem to recruit similar proteins, the key to the CDM1 enigma may reside in the expression of certain proteins during fetal development. DNA methylation causing the malfunction of regulatory elements such as insulators may also have an important part to play in CDM1.

The data presented in this report indicate that there are now at least two differences at the molecular level distinguishing CDM1 from DM1: methylation of the mutant DMPK allele and increased levels of mutant DMPK mRNA. These two parameters provide important tools in the diagnosis of CDM1 in fetuses, tissues and cell cultures when a clinical diagnosis is not possible. The exact implications of these abnormalities in generating the severe congenital form of DM1 are yet to be understood.

2.7. Acknowledments

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[1]Abbreviations used are: DMPK, myotonic dystrophy protein kinase; CDM1, congenital myotonic dystrophy type 1, DM1, myotonic dystrophy type 1; DM2, myotonic dystrophy type 2.

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2.9. Figure legends

Figure 1. Northern blot analysis of DMPK RNA from differentiated myoblasts using three commonly used extraction buffers. (A) RNA was extracted from Normal human myoblasts and two DM1 myoblasts populations with respectively 750 and 1200 repeats. Guanidium thiocyanate buffer was used for lanes 1-3, Trizol reagent was used for lanes 4-6 and proteinase K lysis buffer was used followed by Trizol extraction for lanes 7-9. The blot was hybridized first with a DMPK probe followed by a cyclophilin probe for normalization. Ten micrograms of total RNA was loaded on gel for the proteinase K extraction, while 15 μg were loaded for the other two methods. One representative experiment is shown. **(B)** Quantifications of normal (N) and mutant (Mut) transcript expression were done as described in the experimental procedures section. Each graph depicts the mean ± SEM of three independent experiments (n = 3). *, **, significantly different from normal DMPK mRNA at $P<0.03$ and $P<0.01$ respectively; n.s., not significantly different (by ANOVA).

Figure 2. Representation of a methylation-sensitive restriction map and Southern blot analysis. (A) Restriction map of the 1.8 kb region of interest of the *DMPK* gene. The probe used overlaps the upstream Sac-I to the downstream Sac-II fragment of the *DMPK* gene. The methylation status of Sac-II sites are indicated. **(B)** Southern blot performed on Normal (A), DM-750 (B) and DM-3200 (C) cells. Restriction enzymes used for genomic DNA digestions are the following: Sac-I (SI), Sac-II (SII), Hind-III (H) and Hha-I (Hha). The asterisk (*) indicates the DNA fragment containing the aberrantly methylated downstream Sac-II site in DM-3200 myoblasts. Bands at 4.0 kb and 11.4 kb represent full-length (Sac-I to Hind-III) mutant transcripts of DM-750 and DM-3200 myoblasts respectively. Bands at 1.8 kb represent the full-length normal transcripts in all myoblasts.

Figure 3. Accumulation of mutant DMPK mRNA in CDM1 myoblasts. (A) A Northern blot was performed using proteinase K for proper extraction of total RNA. Congenital DM1 myoblasts with 3200 and 3700 repeats and total RNA extracted from the quadriceps of a CDM1 foetus with 3200 CTG repeats show prominent accumulation of mutant (Mut) over normal (N) DMPK transcripts. **(B)** Quantifications were performed as in figure 1B and described in the experimental procedures section. Bars represent standard error (n = 2). **, significantly different from normal DMPK mRNA at $P<0.01$ (by ANOVA).

Figure 4. Normal and mutant DMPK transcripts are fully polyadenylated. (A) Polyadenylation status of DMPK RNA was achieved by digesting myoblasts with proteinase K and performing either a total RNA extraction (T) with Trizol reagent or polyA(+) mRNA purification (A+) with oligo (dT) cellulose. The fraction unbound to the oligo (dT) cellulose (A-) was precipitated and loaded on gel. No signal was present in this fraction. **(B)** Agarose gel prior to transfer stained with Ethidium bromide. No rRNA bands are visible in the PolyA(+) lanes. **(C)** Quantifications were performed as in figure 1B and described in the experimental procedures section. Bars represent standard error (n = 3). **, significantly different from normal DMPK mRNA at $P<0.01$; n.s., not significantly different (by ANOVA).

Figure 5. Mutant DMPK transcripts in all cells are transcribed to the same rate as normal transcripts. (A) Northern blot performed on various cells using a ^{32}P-dCTP-labelled $(CTG)_{80}$ probe. Mutant (Mut) and normal (N) DMPK transcripts are indicated with their expected size. **(B)** Nuclear run-on experiments were performed as described in the experimental procedures section using ^{32}P-ATP. Labelled nascent RNA from the nucleus of myoblasts was isolated and hybridized independently to the dot blots. Dot blots were made in duplicate and contained from top to bottom: the $(CTG)_{80}$ probe, DMPK probe (ref. Fig. 2B), pBluescript vector DNA and cyclophilin cDNA. Experiments were done in parallel using the transcription inhibitor α-amanitin as negative controls (data not shown). No

significant differences were measured between normal and mutant alleles transcription levels by ANOVA (data not shown).

Figure 6. Mutant DMPK RNA in CDM1 myoblasts is more stable than normal transcripts. DMPK RNA stability was assessed using actinomycin D as described in materials and methods. **(A)** Northern blot showing the decay of normal (N) and mutant (Mut) DMPK transcripts in various myoblasts. 18s rRNA expression was preferred to cyclophilin for normalizations because of its prolonged half-life in cells. **(B)** Quantifications of normal (N) and mutant (Mut) transcript expression were performed as described in the experimental procedures section. Graphs depict the log of relative expression for each time point. Half-life of transcripts was established using the equation of the linear trend line for a relative intensity of log (0.5). Time points represent the average of measurements ± standard error (n = 6 for Normal, n = 4 for DM-3200 and n = 2 for DM-750).

Figure 7. Elevated levels of mutant DMPK mRNA in congenital myoblasts occur at day 1 of differentiation. **(A)** Northern blot showing induction of DMPK and cyclophilin expression after serum deprivation. Mutant (Mut) and normal (N) DMPK transcripts are indicated. Cells in proliferation (Time = 0) were harvested at 90% confluence prior to serum deprivation. **(B)** Quantifications of normal (N) and mutant (Mut) transcript expression were performed as described in the experimental procedures section. Bars represent standard error (n = 3 for normal, DM-1200 and DM-3200 and n = 2 for DM-750). *, **, significantly different from normal and/or mutant DMPK mRNA at T=1 for each panel with $P<0.03$ and $P<0.01$ respectively (by ANOVA).

2.10. Figures

FIGURE 1

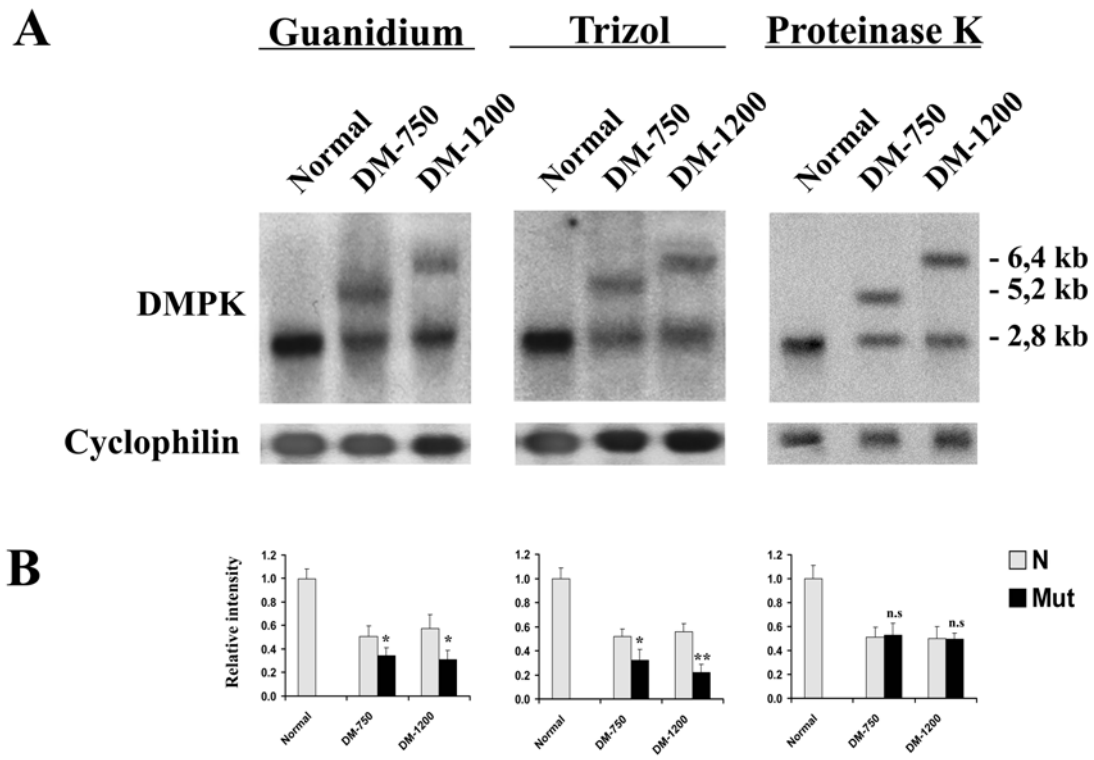
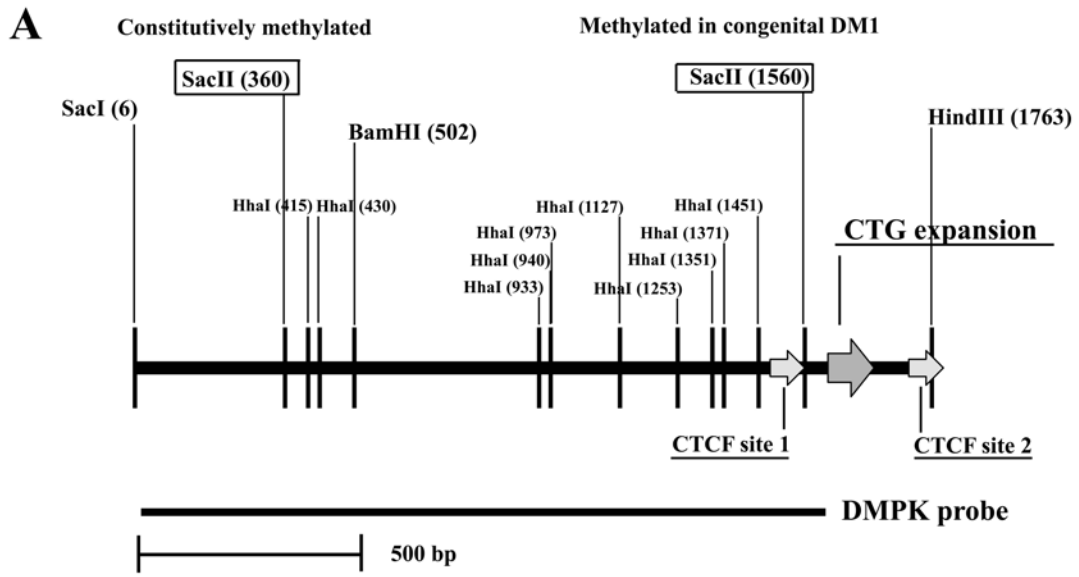


FIGURE 2


A
Constitutively methylated
Methylated in congenital DM1
SacII (360)
SacII (1560)
SacI (6)
BamHI (502)
HindIII (1763)
HhaI (415)
HhaI (430)
HhaI (1127)
HhaI (1451)
HhaI (973)
HhaI (1371)
CTG expansion
HhaI (940)
HhaI (1351)
HhaI (933)
HhaI (1253)
CTCF site 1
CTCF site 2
DMPK probe
500 bp

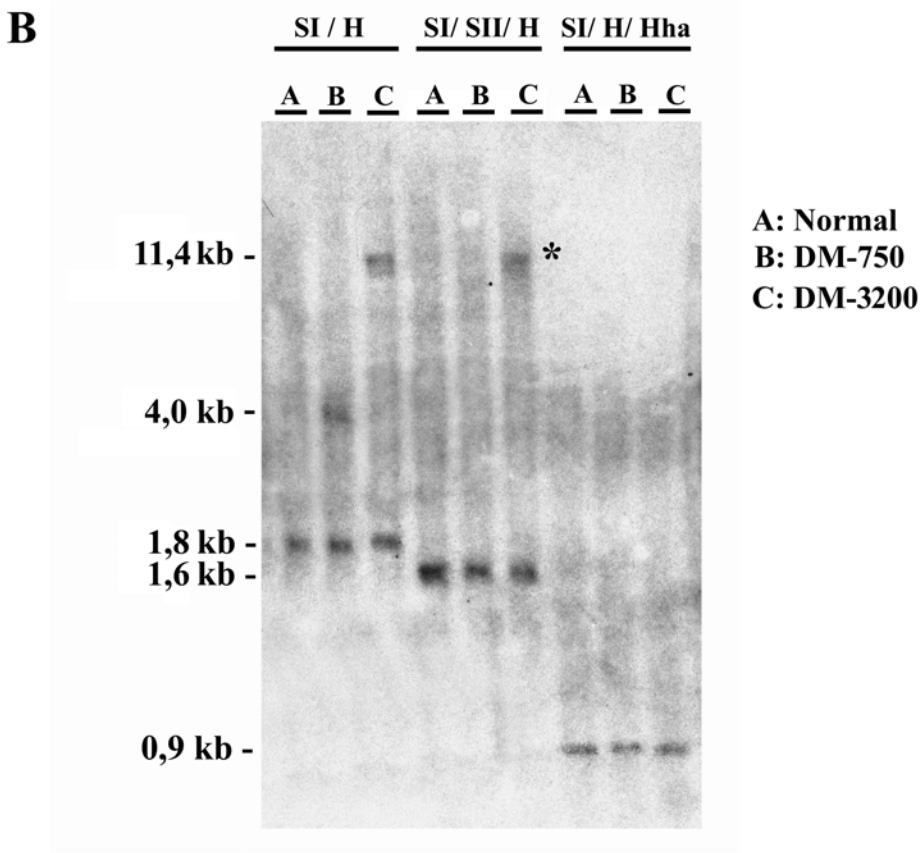

B
SI / H
SI/ SII/ H
SI/ H/ Hha
A B C A B C A B C
11,4 kb -
4,0 kb -
1,8 kb -
1,6 kb -
0,9 kb -
*
A: Normal
B: DM-750
C: DM-3200

FIGURE 3

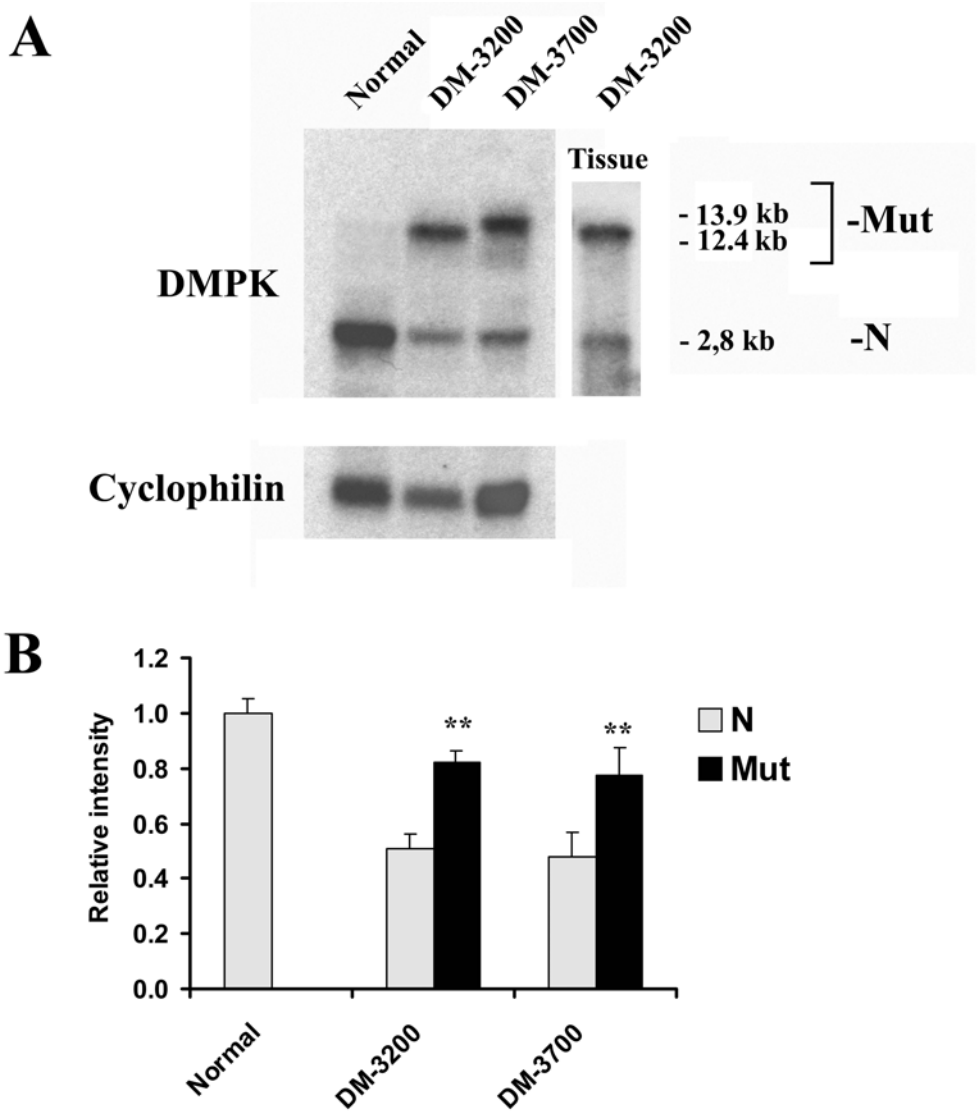

A
Normal
DM-3200
DM-3700
DM-3200
Tissue
DMPK
- 13.9 kb
- 12.4 kb
-Mut
- 2,8 kb
-N
Cyclophilin
B
Relative intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
N
Mut
Normal
DM-3200
DM-3700

FIGURE 4

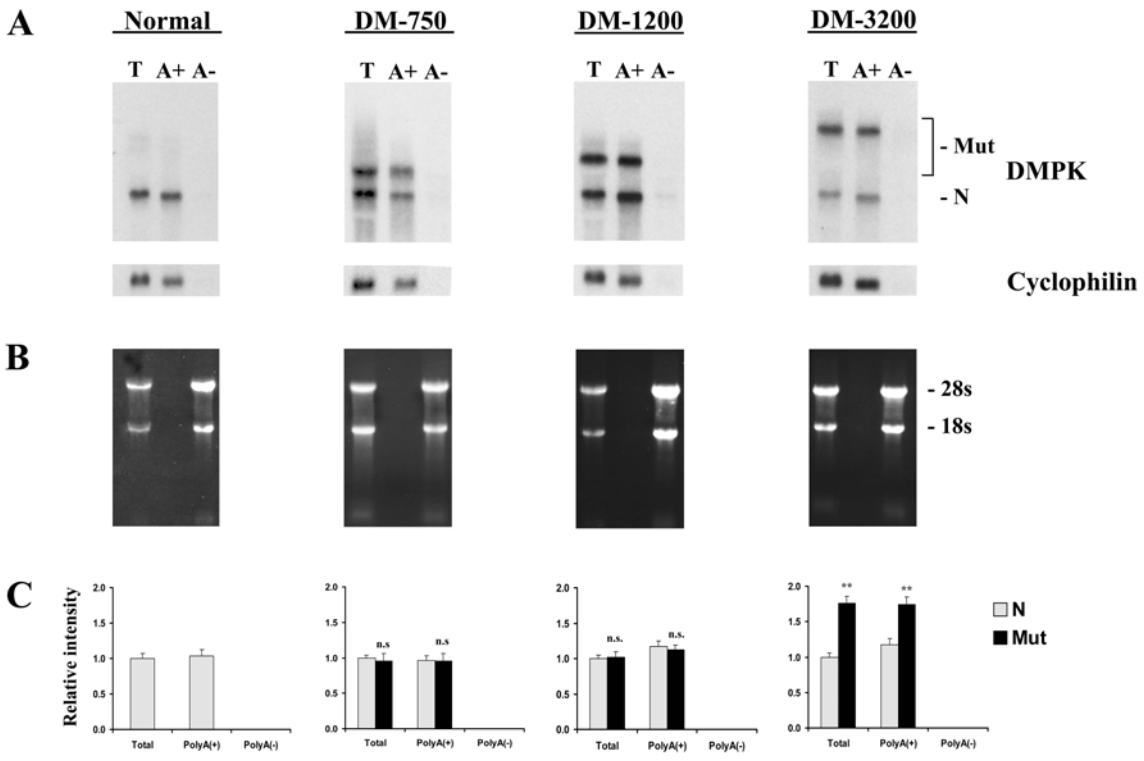

A
Normal
DM-750
DM-1200
DM-3200
T A+ A-
- Mut
- N
DMPK
Cyclophilin
B
- 28s
- 18s
C
Relative intensity
Total
PolyA(+)
PolyA(-)
n.s
**
N
Mut

FIGURE 5

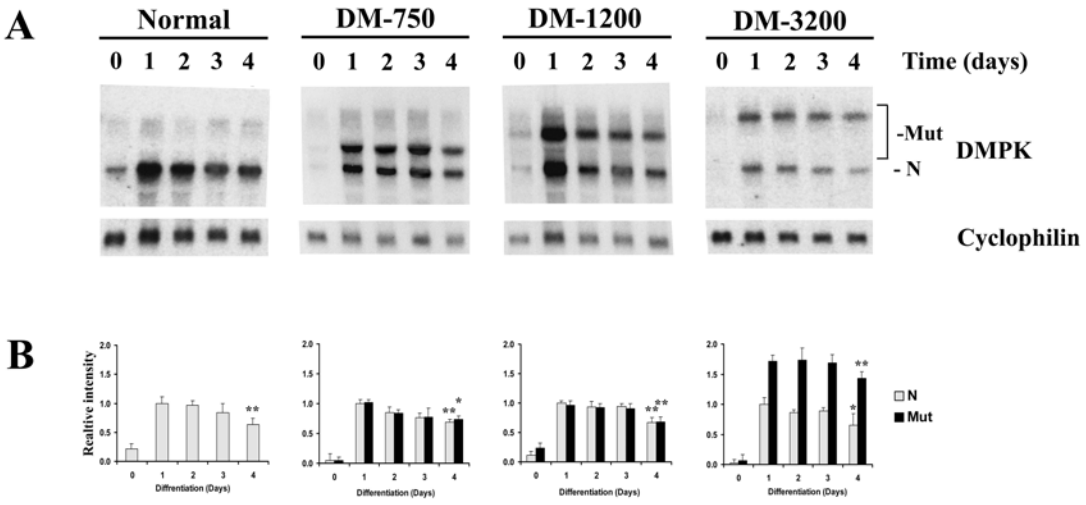

A
Normal
DM-750
DM-1200
DM-3200
0 1 2 3 4
Time (days)
-Mut
- N
DMPK
Cyclophilin
B
Realtive intensity
Diffrentiation (Days)
Differentiation (Days)
N
Mut

FIGURE 6

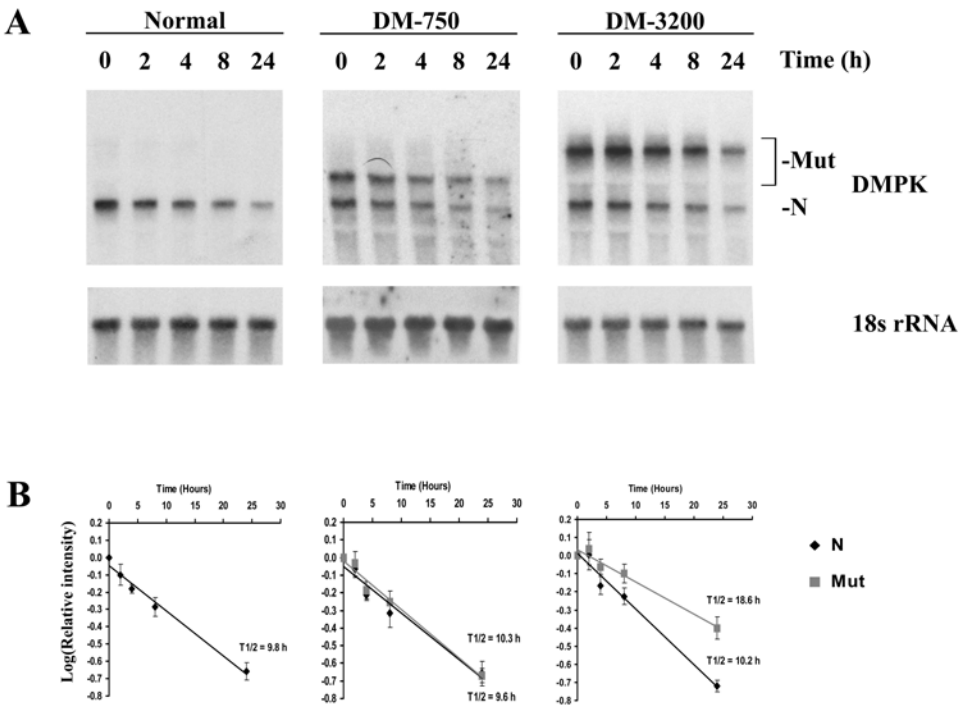
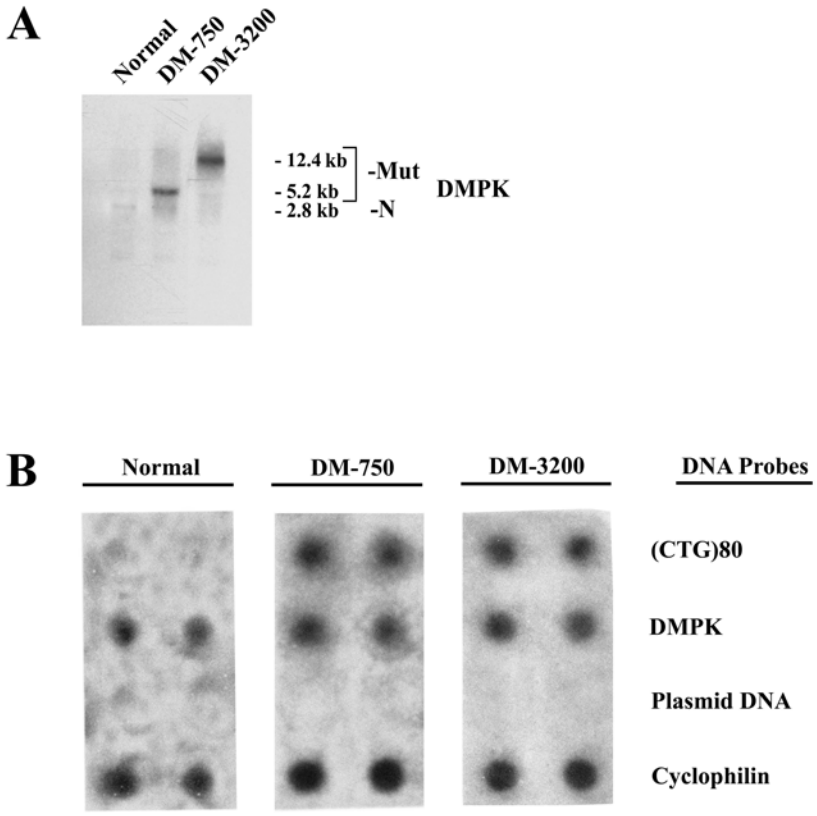

A
Normal
DM-750
DM-3200
0 2 4 8 24
0 2 4 8 24
0 2 4 8 24
Time (h)
-Mut
-N
DMPK
18s rRNA
B
Time (Hours)
Log(Relative intensity)
T1/2 = 9.8 h
T1/2 = 10.3 h
T1/2 = 9.6 h
T1/2 = 18.6 h
T1/2 = 10.2 h
N
Mut

FIGURE 7


A
Normal
DM-750
DM-3200
- 12.4 kb
- 5.2 kb
- 2.8 kb
-Mut
-N
DMPK
B
Normal
DM-750
DM-3200
DNA Probes
(CTG)80
DMPK
Plasmid DNA
Cyclophilin

CHAPTER III

Viral vector producing antisense RNA restores myotonic dystrophy myoblast functions

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Running title: Targeting of mutant DMPK transcripts

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3.1. Résumé

La dystrophie myotonique de type I (DM1) est causée par une expansion du trinucleotide (CTG) situé dans la région 3' non-codante du gène de la dystrophica myotonica protéine kinase (DMPK). Il n'y a présentement aucun traitement efficace pour cette maladie. Les résultats actuels dans la littérature suggèrent qu'un déséquilibre dans l'homéostasie de cet ARN pourrait être responsable d'un grand nombre de symptômes de la maladie. Ceci indique qu'un ciblage spécifique des ARNm mutés de DMPK est essentiel au développement d'une thérapie génique pour la DM1. Dans ce travail nous avons développé un rétrovirus exprimant un ARN antisens complémentaire à une région de 149 paires de bases de l'ARNm de DMPK. Lorsque cet antisens est exprimé dans des myoblastes DM1, nous observons une diminution préférentielle des messagers mutés, ainsi qu'un rétablissement de la capacité de ces myoblastes à fusionner et à capturer le glucose, deux paramètres qui sont altérés dans myoblastes DM1. De plus, nous observons une diminution marquée de la protéine CUG-BP1 qui est anormalement élevée dans les noyaux des myoblastes DM1. Nous démontrons donc pour la première fois le potentiel des ARNs antisens à rétablir un phénotype normal dans les cellules DM1, ce qui ouvre la porte à une thérapie potentielle.

3.2. Abstract

Myotonic dystrophy (DM1) is caused by the expansion of a trinucleotide repeat (CTG) located in the 3'untranslated region of the myotonic dystrophy protein kinase gene, for which currently there is no effective treatment. The data available suggest that misregulation of RNA homeostasis may play a major role in DM1 muscle pathogenesis. This indicates that the specific targeting of the mutant DMPK transcripts is essential to raise the rationale basis for the development of a specific gene therapy for DM1. We have produced a retrovirus which expresses a 149-bp antisense RNA complementary to the (CUG)13 repeats and to the 110-bp region following the repeats sequence to increase the specificity. This construct was introduced into human DM1 myoblasts, resulting in a preferential decrease in mutant DMPK transcripts, and effective restoration of human DM1 myoblast functions such as myoblast fusion and the uptake of glucose. It was previously shown that delay of muscle differentiation and insulin resistance in DM1 are associated with misregulation of CUGBP1 protein levels. The analysis of CUGBP1 levels and activity in DM1 cells expressing the antisense RNA indicated a correction of CUGBP1 expression in infected DM1 cells. We therefore show that current antisense RNA delivered *in vitro* using a retrovirus is not only capable of inhibiting mutant DMPK transcripts, but also can ameliorate dystrophic muscle pathology at the cellular levels.

Keywords: myotonic dystrophy, myoblast, gene therapy

3.3. Introduction

Myotonic dystrophy (DM1) is the most common form of inherited neuromuscular disorder in adults with a global incidence about 1 in 8500 individuals[1]. The genetic basis for this autosomal dominant disease is an expanded trinucleotide repeat (CTG) located in the 3'-untranslated region (3"UTR) of the dystrophy myotonic protein kinase (DMPK) gene[2-4]. At the molecular levels, the DM1 phenotype is most likely caused by a complex molecular pathogenesis, including deficiency of myotonic dystrophy myotonin kinase (DMPK) protein[5-7], haplo-insufficiency of a neighboring homeobox gene (particular the DM locus-associated homeodomain protein (DMAHP/Six 5 gene)[8] and the WD-repeat gene (DMWD)[9], and a trans-dominant misregulation of RNA homeostasis[10-16]. Recent experiments from transgenic mice, expressing an untranslated expanded CUG repeat under the control of the human skeletal actin promoter, showed that expanded CUG repeats are sufficient to generate DM1 muscle phenotype[17]. These data suggest that misregulation of RNA homeostasis may play a major role in DM1 muscle pathogenesis. This indicates that the specific targeting of the mutated DMPK transcripts is essential to raise the rationale basis for the development of a specific gene therapy for DM1, at least for muscle weakness and wasting.

Gene silencing has been achieved with varying efficiencies using antisense DNA or RNA, ribozymes, and more recently siRNAs[18-21]. The antisense RNA technology utilizes ribonucleic acids which, like antisense oligonucleotides, bind by complementary interactions with a specific target mRNA (in this case forming RNA:RNA hybrids) and subsequently either block one of the several steps involved in the translation of a targeted protein or induced destruction of the target RNA most likely by activating an endonuclease activity following deamination of dsRNA by an adenosine deaminase[22,23]. Expression vectors producing antisense RNA have major advantages over the oligonucleotides in that, since vectors can synthesize the antisense RNA continuously inside the cell after a single administration, it would have a longer duration of action.

We have therefore designed, constructed and evaluated the effect of an antisense RNA directed complementary to (CUG)13 repeat sequence and to the 110-bp sequence, following the expansion, using a vector derived from Moloney murine leukemia virus (MMLV), for use in human DM1 myoblasts. We demonstrated that expression of antisense CUG in DM1 muscle cells is able to correct delay of muscle fusion and insulin resistance via normalization of CUGBP1 levels.

3.4. Materials and methods

Human skeletal myoblasts

Human myoblasts were maintained and expanded at 37°C in the proliferative medium MCDB120 supplemented with 15% fetal bovine serum. The percentage of myogenic cells in the culture was determined by immunocytochemistry analysis using anti-desmin antibody as described previously.[24–26] Southern blot analysis showed a length of CTG repeats of about 750 in DM1 myoblasts.[25] For myoblast differentiation, cells were subsequently cultured in a-MEM supplemented with 10 mg/ml insulin, 10 ng/ml epidermal growth factor and 0.5 mg/ml BSA.[28] Cells were grown in 5% CO2 humidified incubator.

Vector construction and production

Construction of pLXie (CUG)13 antisense RNA: A 170-bp DNA fragment was isolated from genomic DNA by PCR using primers 406 and 409 (Mahadevan), cloned into TA cloning kit. This fragment contains a 22-bp region preceding the repeats, 13 CTG repeats and a 110-bp region following the repeats in the 30UTR of the DMPK mRNA. This fragment was then excised by digestion with BsaMI/Xho and cloned into the HpaI and Xho restriction sites in pLXie. The final construct expresses an antisense RNA complementary to (CUG)13 repeats and to the 110-bp region following the repeats.

Construction of pLXie 50UTR antisense: A 5-kb BamHI fragment from the 50end of the human DMPK gene cloned into pUC18 was a gift from Dr MS Mahadevan (Acc # L08835). A 857-bp DNA fragment (_2169 to _1311) in the 50UTR of the DMPK gene was excised by digestion with XmnI/AflIII, blunted and cloned into the blunted EcoRV restriction site of pcDNA3. The orientation of the fragment was determined by DNA sequencing. The antisense was excised from pcDNA3 by digestion with EcoRI/XhoI and cloned in pLXie at the EcoRI and XhoI restriction sites.

All the constructs were verified by direct sequencing (Perkin Elmer, Foster City, CA, USA). To produce infectious vectors, 293-gpg cells at a density of 10–12X10^6 per 10-cm culture dishes were transfected with 1 mg of pLXie, by using Transfectam (Qiagen). Vectors were harvested at day 7 after the transfection period, in a medium that has been in contact with the cells for 4 days.

Transduction of human primary myoblasts

The supernates containing the pLXie vector were pretreated with 80 mg/ml polybrene, and cells at 60–70% confluence were transduced with pLxie vectors by centrifugation at 800g for 30 min at 20^{o}C. This step was repeated 18 h later. The transduced cells were washed and incubated in MCDB120 for 24–36 h. Cells were then trypsinized and GFP-positive cells were isolated by fluorescence-activated-cell-sorting (FACS). GFP-positive cells were grown in a proliferative medium as described above.

Cell analysis

Cell proliferation was determined by MTT assay.[35] The index of fusion was determined by counting the number of nuclei in multinucleated myotubes and it was expressed as a percentage of the total number of nuclei. 2-deoxy-[3H]deoxyglucose glucose transport: 2-Deoxyglucose uptake was performed according to the procedure described by Sarabia et al,.[36] Myotubes were preincubated for 24 h in serum-free media depleted in insulin and containing 5.5 mM glucose. The cells were then incubated with or without the desired concentration of insulin (0–1 mM) for 45 min at 371C and then, rinsed once with glucose-free HEPES-buffered saline solution and subsequently incubated for 8 min with 10 mM 2-deoxy-[3H]-glucose. After washing, cells were lyzed in 0.05 N NaOH, and the radioactivity was determined by scintillation counting. Non-carrier-mediated uptake was determined in parallel determinations in the presence of 10 mM cytochalasin B and was subtracted from all experimental values. Protein content was determined by the method of Bradford using

the Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA, USA). Glucose transport is expressed throughout in pmol/min/mg protein.

Northern blot analysis

Total RNA was extracted using Trizol reagent (Life Technologies, Inc.) on whole cells. RNA concentrations were determined spectrophotometrically by measuring the A260. In total, 12 mg of RNA was separated on 1.2% agarose gels containing 0.66 M formaldehyde, and transferred to GeneScreen Plus membranes. The recovery of RNAs was determined by hybridization with a 32Plabeled cyclophilin DNA probe as described.32 DMPK probe was generated by random priming of 25–50 ng of the DMPK cDNA (BglII–SacI fragment) and hybridization was done overnight at 68^{o}C in hybridization buffer (2% SDS/10% dextran sulfate/1XSSPE/ 10 mg/ml salmon sperm DNA/ 2% Denhart's). Quantitation of relative RNA levels of autoradiograms was determined by densitometry (AlphaImager scan from Alpha Innovatech Corp.). All values were normalized to cyclophilin signal.

Western analysis

To determine CUGBP1 levels in muscle cells, whole cell protein extracts were prepared from normal, DM1 and DM1 myoblasts infected with recombinant retrovirus expressing antisense CUG13. Proteins were separated by 10% polyacrylamide gel electrophoresis with SDS and transferred onto nitrocellulose membrane. CUGBP1 was detected with polyclonal antibodies against CUGBP1 (1:1000) as described.14 To determine protein loading, the same membrane after CUGBP1 detection was reprobed with antibodies against β-actin, and CUGBP1 levels were calculated as ratio to β-actin.

UV-crosslinking analysis

RNA probe containing 123 CUG repeats was synthesized in in vitro transcription assay (Roech Molecular Biochemical) using 32P-UTP. Proteins from normal, DM1 and DM1 myoblasts infected with recombinant retrovirus expressing antisense CUG repeats were incubated with radioactive RNA for 30 min at room temperature, and then subjected to UV treatment for 5 min as described.[33]

3.5. Results

Construction of recombinant retrovirus expressing (CUG)13 Antisense RNA

Initial experiments to inhibit DMPK expression were performed with 3 antisense oligonucleotides (ODNs), one directed to the 5'UTR, the second against the 3'UTR (between the stop codon and the beginning of the expansion) and the third against a (CUG)13 repeats, at various concentrations in cell extracts. We identified the (CUG)n sequence located in the 3'UTR of the DMPK transcripts as a potential antisense target site whereas no inhibition was observed with the two other ODNs (data not shown). Based on these results, we tested the retrovirus containing a 170-bp (CUG)13 cDNA fragment under the control of the 5'LTR (Fig. 1). Two other antisenses were performed, one against a 875 bp region in the 5'UTR of the DMPK mRNA and the other against a 249 bp fragment in 3'UTR of the DMPK mRNAs (between the stop codon and the first CUG). All these retrovirus also contain the EGFP gene for cell selection (Fig.1).

Human normal and DM1 myoblasts were infected with recombinant retrovirus, and GFP-positive cells were selected by FACS. Cells were grown in permissive medium until confluence and then, were shifted in differentiating medium. In these conditions, more than 99% of the cells were GFP-positive. The levels of transgene expression was determined by Northern blot. As shown in Fig. 2A, the 5'UTR and (CUG)13 antisense RNAs were expressed at high levels in human DM1 cells whereas no expression was found for the 3'UTR antisense RNA, at day 5 of differentiation. In further experiments, we analyzed the levels of DMPK mRNAs in human DM1 cells expressing the three antisenses (Fig.2B). In normal myoblasts, the probe detected one 3.3 kb mRNA which is the expected size of the DMPK mRNA. In DM1 myoblasts the probe detected a 3.3 kb mRNA and a higher RNA band of approximately 5.6 kb which correspond to the expanded transcripts. In DM1 cells infected with the antisense RNA against the repeat sequence, we found a 80 and 50% decrease in the levels of the mutant and normal DMPK mRNAs, respectively. In contrast,

no effect of antisense RNAs against the 5'UTR and the 3'UTR on DMPK mRNAs was observed. No effect on DMPK mRNAs was observed with a retrovirus expressing EGFP alone (not shown). From these data, we concluded that (CUG)13 antisense vector inhibits both normal and mutant DMPK mRNA, but there was a more important inhibition of mutant than normal DMPK mRNAs. Because the 5'UTR and 3'UTR antisense RNA have no effect on DMPK gene expression, these cells were used as control in further experiments.

Restoration of DM1 myoblast differentiation by antisense RNA.

DM1 and normal muscle cells used in this study were purified by cell sorting using a neural cell adhesion molecule (N-CAM) antibody as previously described[25]. More than 90% of DM1 and normal cells were desmin-positive indicating that they are myoblasts. The proliferating infected DM1 myoblasts are typically spindle shaped and have a very similar morphology to the proliferating normal myoblasts or uninfected DM1 myoblasts. The growth of normal, DM1, and DM1 infected myoblasts in proliferative medium was determined by MTT assay. As shown in fig. 3 the growth of normal, DM1 and infected DM1 myoblasts were very similar with a doubling time about 36 h. No effect of the two control antisenses was observed on myoblast proliferation. This indicates that the antisense vector have no toxic effect on DM1 cells. When DM1 cultures were switched from permissive to non-permissive conditions, the majority of cells ceased DNA synthesis by 48 h, myotube formation began within 3 days of switching to non-permissive conditions, and myotubes continued to increase in size and number over several days. The quality of the differentiation of infected DM1 myoblasts was compared with uninfected DM1 and normal myoblasts. The index of fusion was much lower in DM1 cultures than in the controls (59% vs 90%), and the index of fusion was very similar for normal myoblasts and myoblasts infected with retrovirus producing 5'UTR and 3'UTR antisense RNAs (Table 1). In contrast, the index of fusion of DM myoblasts infected with a retrovirus expressing (CUG)13 antisense RNA was completely restored. In addition, the myotubes formed by both types of cultures showed striking differences. The normal myoblasts formed large branched myotubes with several nuclei, whereas DM1 myotubes were much smaller and

had a relatively small number of nuclei per myotubes, as shown in Fig. 4. Thus, after 5 days of differentiation, control cultures showed an average of 13 nuclei per myotube, whereas DM1 myotubes contained an average of 3.8, 3.5 and 3.5 nuclei per myotube in uninfected, infected with retrovirus producing 5'UTR or 3'UTR antisense, respectively. In infected DM1 cell cultures, myoblasts were much bigger and contains as many nuclei as observed in control cultures (Fig.4).

Insulin resistance is the principal metabolic abnormality associated with the pathology of DM, and we previously showed that insulin resistance is retained in human DM1 myoblast cultures[25]. To determine whether antisense RNA can circumvent defective insulin action in human DM myoblasts, we analyzed the influence of insulin on glucose uptake. In DM1 cells, a dose of 10 and 50 nM insulin produced no stimulatory effect on glucose uptake as previously reported[25]. In contrast, 10 and 50 nM insulin produced a 2-fold increase in the uptake of glucose in DM1 cells producing (CUG)13 antisense RNAs (Fig.5).

Inhibition of CUGBP1 binding activity in infected DM1 cells

It was previously reported that CUGBP1 activity was affected by expansion in DM cell culture models[11-13]. We therefore examine whether the production of antisense RNA could affect its expression. In Fig, 6, Western analysis of protein extracts with antibodies against CUGBP1 showed that protein levels of CUGBP1 was increased in DM myoblasts and the expression of antisense RNA inhibited CUBBP1 induction. Upper band shows cross-reactive protein that was reduced by the antisense, indicating that the antisense could also inhibited other proteins which are cross reactive with CUGBP1 antibodies. It seems that antisense did not affect global protein expression because staining of the membrane with Coomassie blue shows equal amounts of proteins in all three lines. The middle portion of the picture shows reproducible result of UV-cross link assay with RNA probe containing 123 CUG repeats. Only one complex in the position of CUGBP1 is detected with this

probe. In agreement with Western, binding activity of CUGBP1 is induced in DM myoblasts and activity of this protein was inhibited by antisense.

3.6. Discussion

Antisense RNA is a potentially powerful tool for gene silencing. Several issues however have limited wider use of antisense technology. Problems have included a lack of suitable target sequences within a given mRNA caused by RNA secondary folding, which necessitate screening of multiple antisense sequences to identify those that mediate the greatest level of inhibition, and insufficiency delivery in vitro and in vivo. Despite insufficiency delivery in vitro, we found that retrovirus produced high levels of antisense in human myoblasts cultures. Another potential limitation of the use of antisense RNA technology is that they formed RNA:RNA hybrids (dsRNA) in cells which is sufficient to elicit an antiviral response mediated by enzymes such as dsRNA-dependent protein kinase (PKR)[27,28] or 20–50-oligoadenylate synthetase/RNase L,[29,30] which induce apoptosis. Our data do not support an activation of PRK by dsRNA since no apoptosis was observed in infected cells.

DM1 is caused by the expansion of a CTG trinucleotide repeat on chromosome 19q13.3. There are several evidences suggesting that the nuclear accumulation of RNAs from the mutant transcripts[31] may play a major role in the pathogenesis of DM1 muscle. This indicates that the specific destruction of the mutated DMPK transcripts may be essential to restore normal DM1 myoblast functions.

We previously reported that the proliferation rate of diseased myoblasts remains unaffected; however, their differentiation program was altered.[25,32] Here we showed that retrovirus producing (CUG)13 antisense RNA has no effect on the proliferative rate of DM1 myoblasts; however, it restores the differentiation of infected DM1 myoblasts as indicated by the increase in the fusion index, in the mean number of nuclei per myotube as well as in the uptake of glucose. The specificity of these effects is supported by the fact that two other retrovirus producing the GFP protein or an antisense RNA against the 50UTR

have no effect on the levels of DMPK mRNAs nor on the growth and differentiation of DM1 myoblasts. Here we showed that in infected DM1 myoblasts, the (CUG)13 RNA antisense inhibits both the mutant and the normal DMPK transcripts, although at different levels (80 versus 50%, respectively). The strong reduction of the mutated DMPK transcripts by the (CUG)13 antisense could explain the restored differentiation of the infected DM1 myoblasts. This possibility is also supported by the recent report of Timchenko et al[33] showing that alterations in the activity of the CUGBP1 by the mutant DMPK transcripts causes disruption of p21-dependent control of cell cycle arrest, rendering DM1 myoblasts incapable of withdrawing from the cell cycle and fuse to form myotubes. We demonstrated that the misregulation of the CUG-binding protein in DM1 myoblasts was corrected in the antisense-infected DM1 myoblasts, indicating that molecular alterations induced by the mutated DMPK transcript were reversed by the expression of the (CUG)13 antisense in DM1 cells. In addition, it has been suggested that the insulin resistance that occurs in DM1 muscles may be the consequences of alteration in the splicing of insulin receptor pre-mRNA caused by the increased expression of CUG-binding proteins.[34] We showed that the defective action of insulin on glucose uptake retained in DM1 muscle cell cultures was also completely restored in (CUG)13 antisense-infected DM1 myoblasts. Thus, decreased levels of mutant DMPK transcripts by the antisense inhibit the abnormal upregulation of the total levels of CUG-binding protein that may counteract the aberrant regulation of the insulin receptor and restore the action of insulin on glucose uptake in DM1 myoblasts. Normalization of CUGBP1 levels in DM1 differentiating myoblasts expressing RNA CUG antisense supports the idea that antisense expression vector is able to correct misregulated pathways caused by mutant DMPK mRNA.

To improve antisense gene therapy, it would be necessary to target specifically the mutant DMPK mRNAs. The most logical approach to target specifically these transcripts would be the design of antisense RNAs, which has a nuclear localization, by using nuclear promoters such as tRNAmeti or Pol III.

Taken as a whole, our data indicate that the decreased levels in mutant DMPK mRNAs are essential to restore DM1 myoblast functions. Our data support a future role for antisense expression vectors as a new gene therapy for myotonic dystrophy. In addition, our data also support that a mRNA encoding for a gene reporter linked to an antisense RNA does not prevent the inhibition of gene expression by the antisense RNA.

3.7. Acknowledgements

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3.9. Figure legends

Figure 1 Schematic representation of the retrovirus constructs. A 857-bp XmnI/AflIII cDNA fragment from the 50UTR of the DMPK and a 149-bp BsaMI/Xho cDNA fragment (containing 13 CTG repeats and 110 bp in the following region) were cloned in antisense orientation in pLXie. This construct also contains the GFP cDNA under the control of an internal ribosomal entry site (IRES) for cell selection.

Figure 2 Expression of the different antisenses RNAs and DMPK mRNAs level in DM1 myoblasts. (a) Antisenses RNA production in the cells were analyzed by Northern blot. In all, 20 μg of total RNA prepared from DM1 myoblasts transduced with pLXie50UTR and pLXie(CUG)13 retrovirus were subjected to electrophoresis and hybridized with a GFP DNA probe. This probe detected mRNAs that correspond to the size of 50UTR-GFP and (CUG)13-GFP mRNAs, respectively. (b) Expression of DMPK mRNAs determined by Northern blot from DM1 myoblasts transduced with pLXie50UTR, PLXie and PLXie(CUG)13, and from uninfected DM1 and normal myoblasts. In all, 20 μg of total RNAs were subjected to electrophoresis and hybridized with a cDNA DMPK probe. Blots were normalized for RNA loading by reprobing with a 32P-labeled probe for cyclophilin. (c) Quantitation of DMPK mRNA expression. The graph shows the mean ± SD of three independent experiments. ** and ***, significantly different from nontransduced DM1 myoblasts, P=0.003 and 0.001, respectively (student's t-test).

Figure 3 Apoptosis profile and proliferative curves of control, DM1 and DM1-infected myoblasts. (a) For annexin V-PE binding assay, cells were transduced with pLXie (CUG)13 retrovirus and then GFP-positive cells were selected by FACScan. Cells were grown for 48 h and stained with annexin V-PE and with 7-AAD. One representative of three different experiments is shown. Right, noninfected DM1 myoblasts; left, DM1 myoblasts infected with pLXie(CUG)13. Samples (10 000 cells) were analyzed by

FACScan. (b) Cells were grown in proliferative medium for 9 days. The number of cells at different time was quantified by MTT assay. Values represent the mean±S.D. of three independent experiments. Each experiment was made in triplicate. No significant difference was observed between the growth of normal and DM1 cells nor between the growth of DM1 and transduced cells. (Student's t-test: for 5'UTR antisense/DM1 cells, P=0.44, 0.41, 0.11, 0.37 and 0.9 at day 1, 2, 3, 5 and 9, respectively. For pLXie/DM1 cells, P=0.23, 0.35, 0.1, 0.48 and 0.2 at day 1, 2, 3, 5 and 9, respectively. For pLXie(CUG)13/DM1 cells, P=0.1, 0.39, 0.11, 0.27, 0.14 at day 1, 2, 3, 5 and 9, respectively.)

Figure 4 Myoblast differentiation in cultures growing in differentiation medium for 5 days. Myoblasts were fixed with 3% paraformaldehyde and stained with DAPI, and examined with a fluorescent microscope at X 25 magnification. Green fluorescence represents the expression of GFP. (a) DM1 myoblasts infected with pLXie 50UTR antisense; (b) DM1 myoblasts infected with pLXie; (c) DM1 myoblasts noninfected; (d) DM1 myoblasts infected with pLXie (CUG)13 antisense RNA; (e) normal myoblasts stained with hematoxylin/eosin.

Figure 5 Comparison of glucose transport activity in normal, DM1 and infected DM1 pLXie (CUG)13) myoblasts. Cells were incubated with aMEM depleted in insulin for 24 h and then incubated in the absence or presence of insulin. Cells were then washed, and 2-deoxyglucose uptake was measured. Results are the mean±SD of three different experiments. Each experiment was performed in triplicate. **, P<0.003; ns, not significant (by student's t-test).

Figure 6 Normalization of total levels of CUGBP1 in DM1 differentiating myoblasts infected with CUG antisense. (a) Western blotting. CUGBP1 levels were determined by Western blot with antibodies against CUGBP1. Elevated total levels of CUGBP1 in DM1 cells were reduced by the infection of cells with antisense CUG. Position of CUGBP1 is

indicated by an arrow. (b) UV-crosslink. The same protein extracts were analyzed by UV-crosslink analysis with RNA probe containing 123 CUG repeats. CUGBP1/CUG123 complex is shown by an arrow. Upper complex in DM1 myoblasts is formed by an unknown protein. CUGBP1 RNAbinding activity is increased in DM1 cell extract. Antisense RNA reduces the activity of CUGBP1 and of the upper CUG-binding protein. The same membrane after UV-crosslink was stained with Coomassie blue to verify protein loading and protein integrity. Expression of antisense does not affect global protein synthesis.

3.10. Figures

FIGURE 1

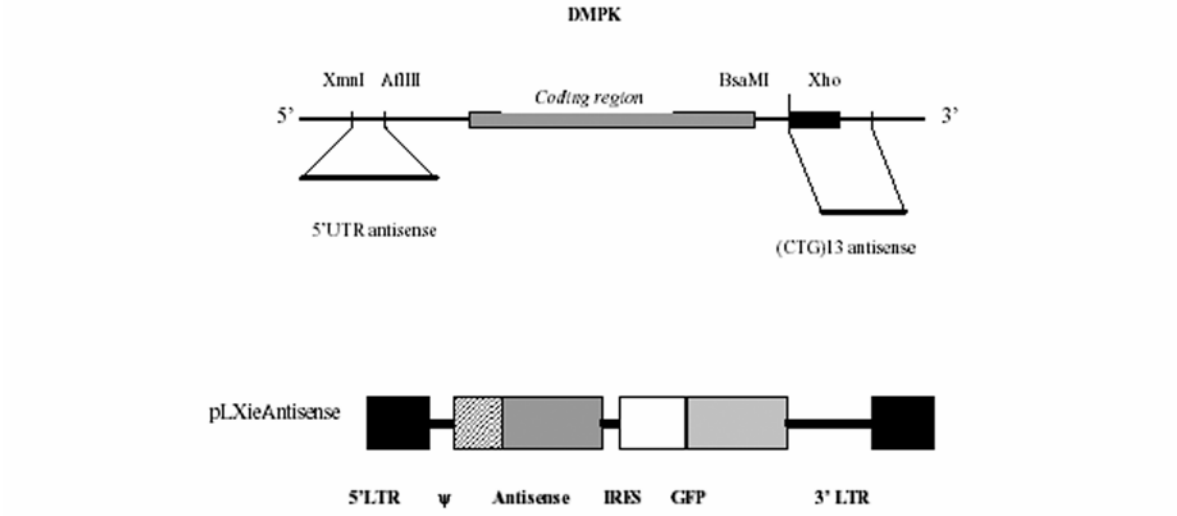


FIGURE 2

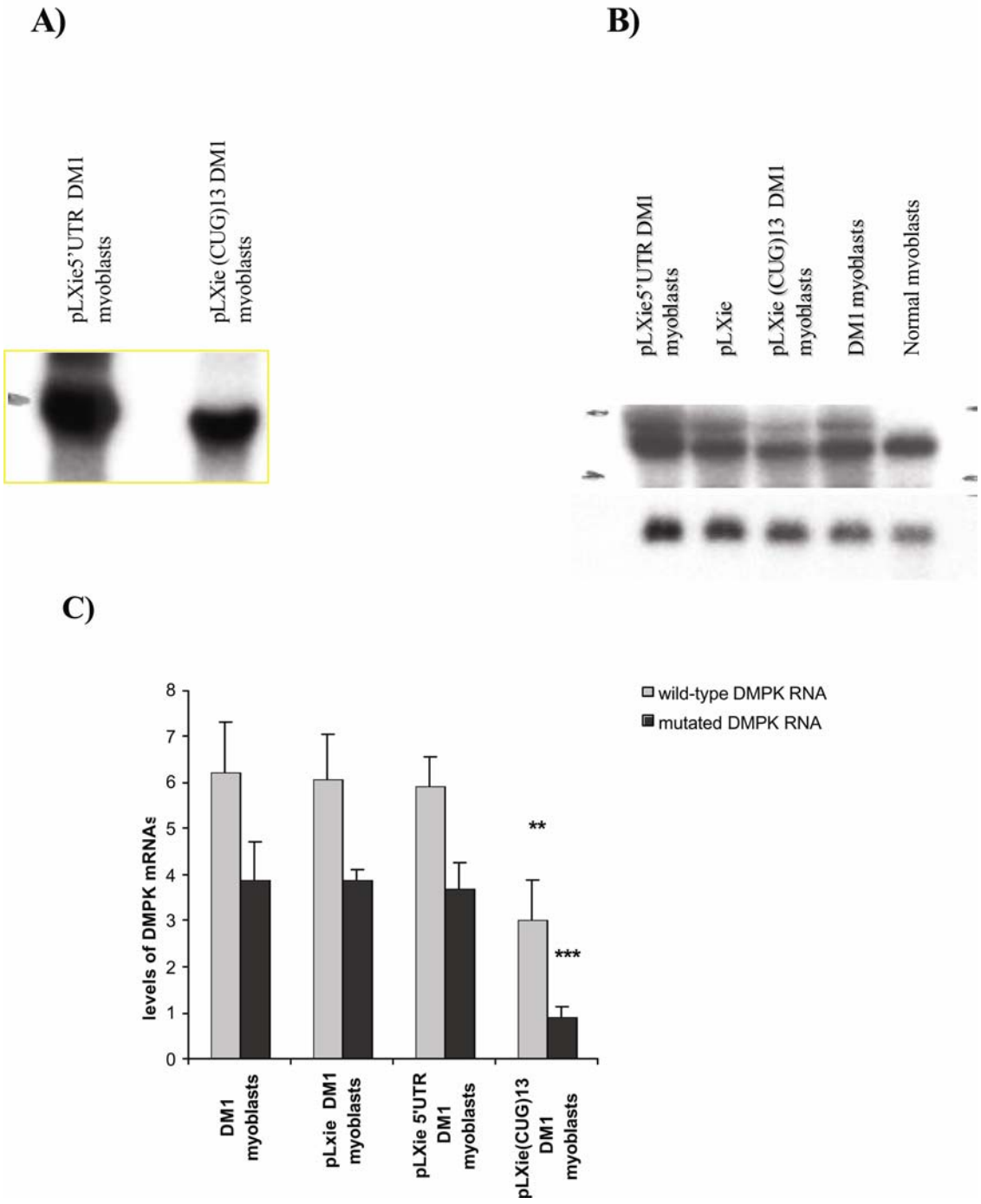

A)
pLXie5'UTR DM1 myoblasts
pLXie (CUG)13 DM1 myoblasts
B)
pLXie5'UTR DM1 myoblasts
pLXie
pLXie (CUG)13 DM1 myoblasts
DM1 myoblasts
Normal myoblasts
C)
wild-type DMPK RNA
mutated DMPK RNA
levels of DMPK mRNAs
0
1
2
3
4
5
6
7
8
**
***
DM1 myoblasts
pLxie DM1 myoblasts
pLXie 5'UTR DM1 myoblasts
pLXie(CUG)13 DM1 myoblasts

FIGURE 3

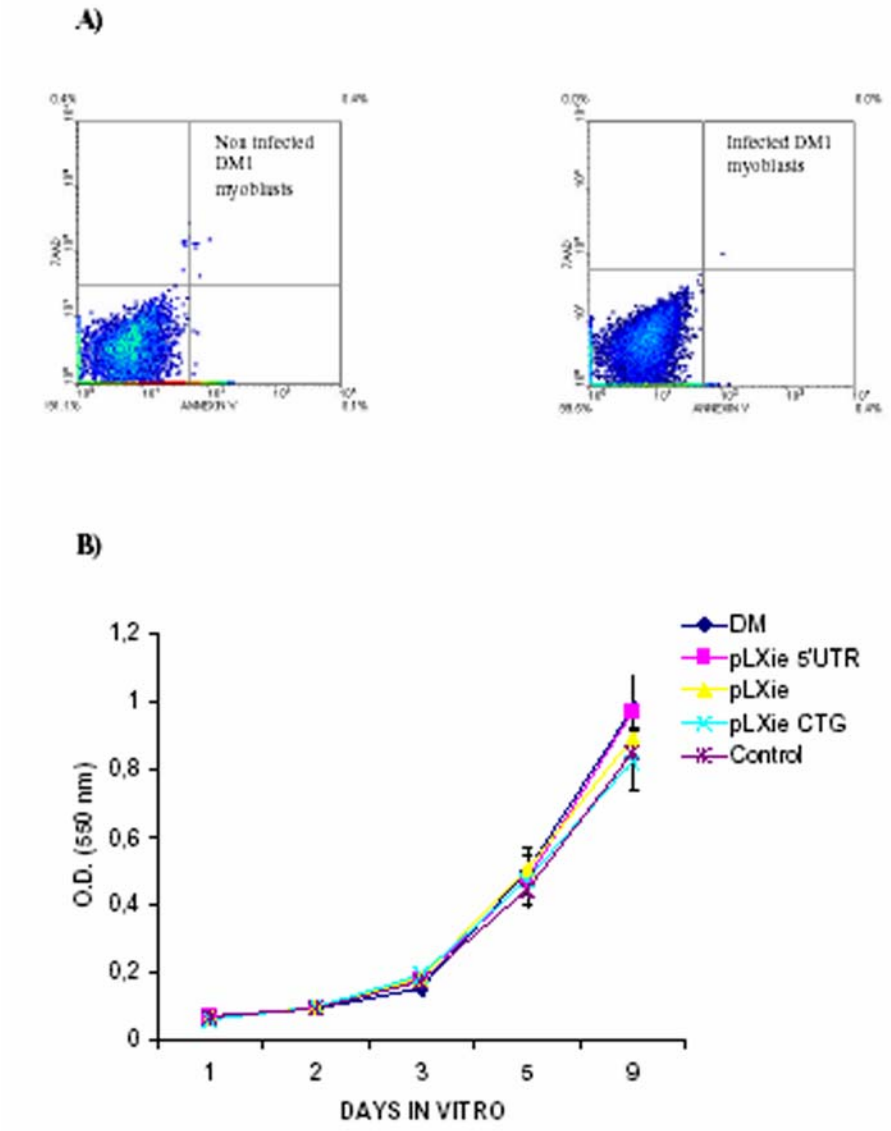

A)
Non infected DM1 myoblasts
Infected DM1 myoblasts
B)
DM
pLXie 5'UTR
pLXie
pLXie CTG
Control
O.D. (550 nm)
1,2
1
0,8
0,6
0,4
0,2
0
1
2
3
5
9
DAYS IN VITRO

FIGURE 4

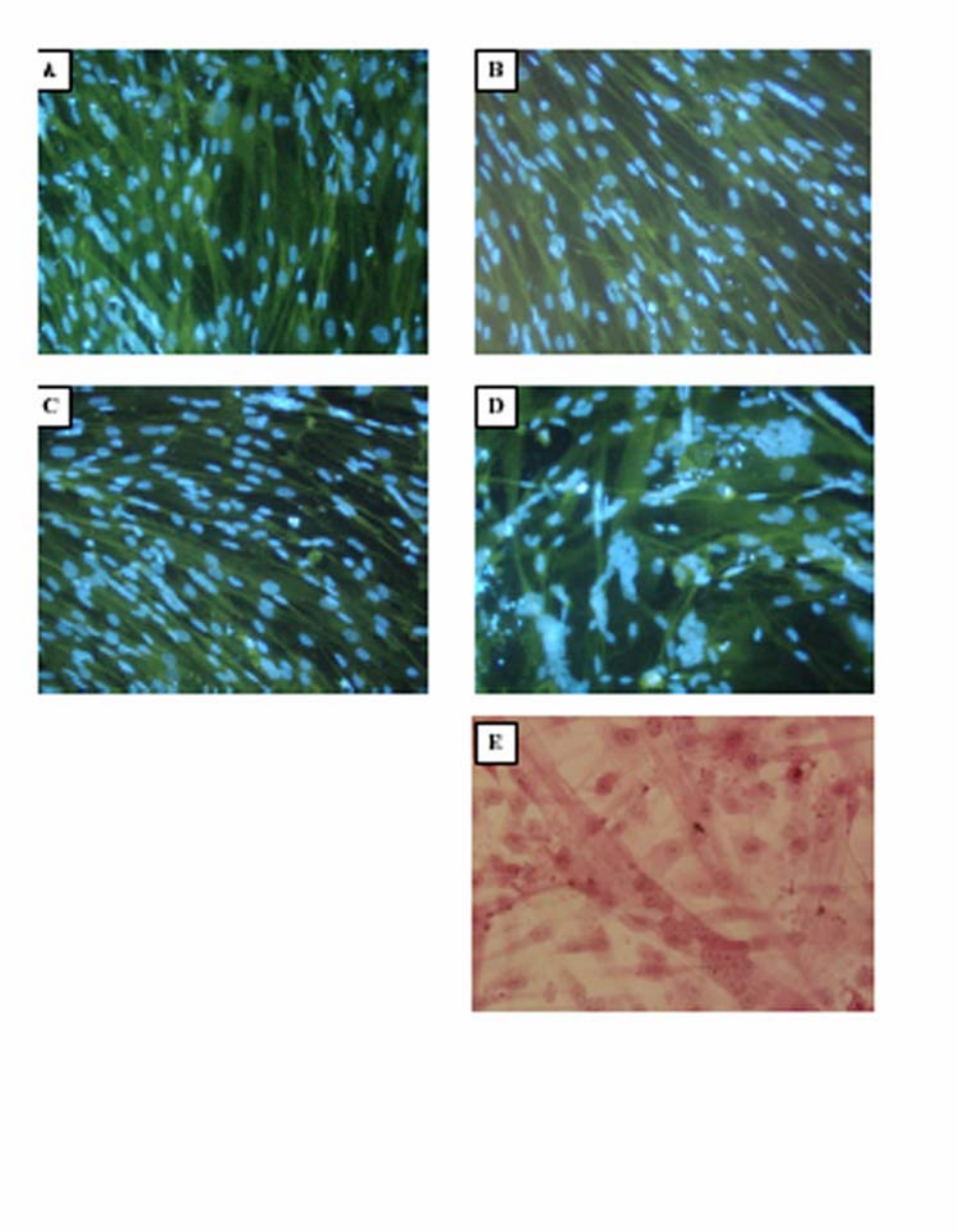


FIGURE 5

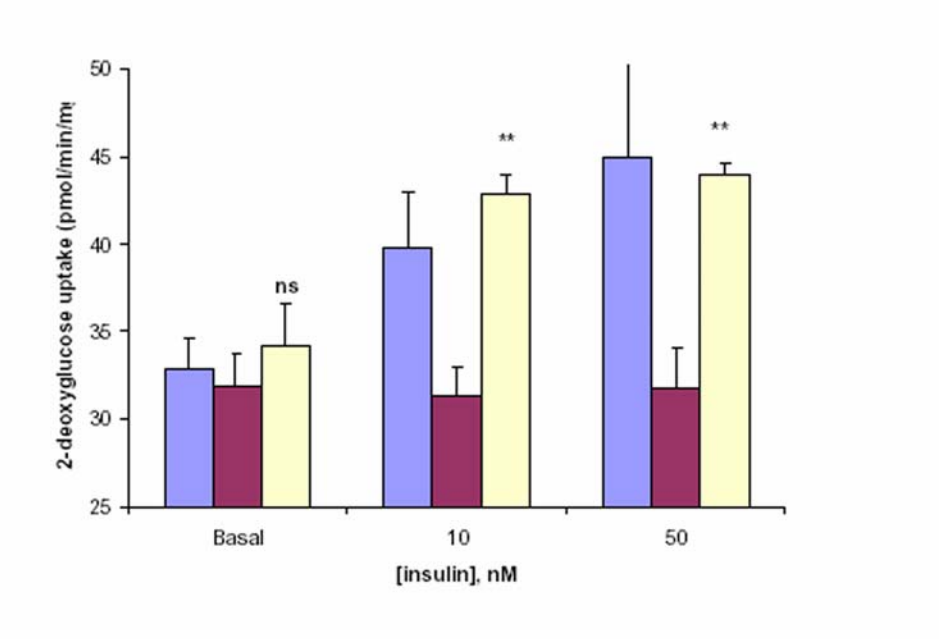
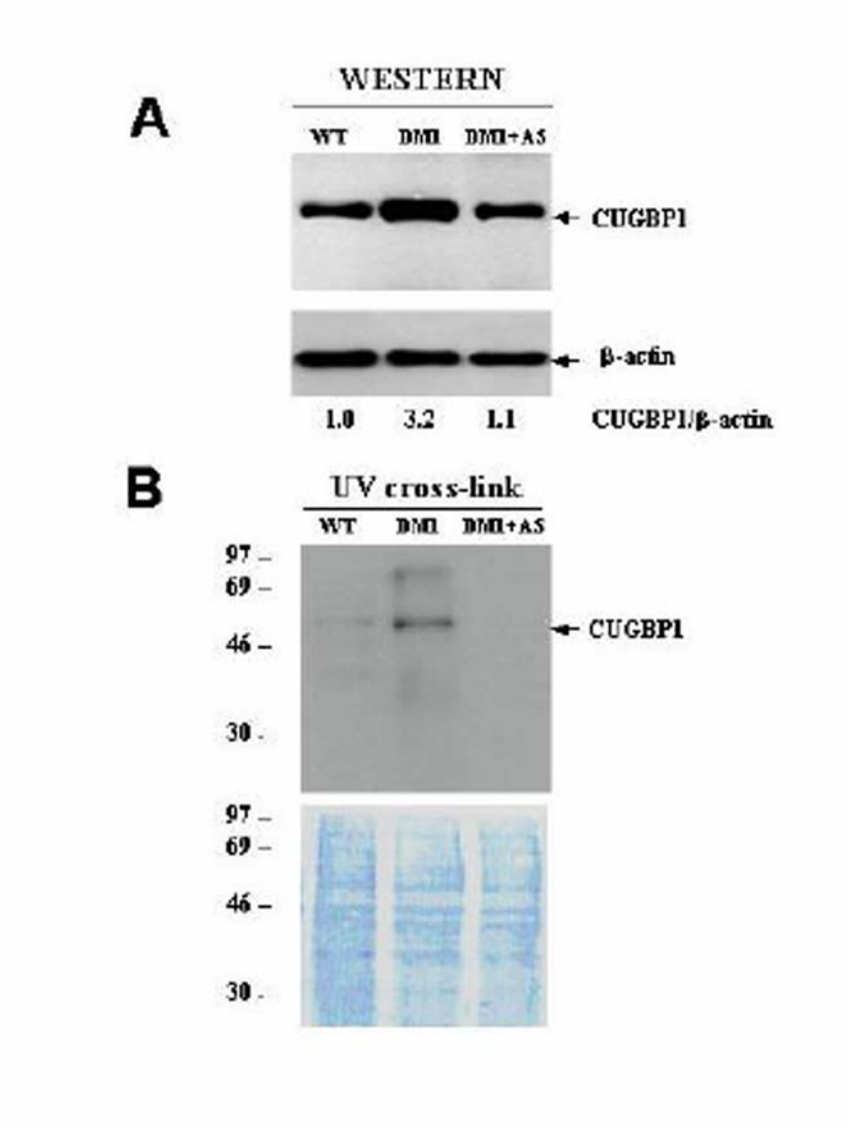

50
45
40
35
30
25
2-deoxyglucose uptake (pmol/min/m
ns
**
**
Basal
10
50
[insulin], nM

FIGURE 6


A
WESTERN
WT
DMI
DMI+AS
CUGBP1
β-actin
1.0
3.2
1.1
CUGBP1/β-actin
B
UV cross-link
WT
DMI
DMI+AS
97
69
46
30
CUGBP1
97
69
46
30

3.11. Tables

Table1 Differentiation of human DM1 myoblasts

Myoblasts	*Index of fusion (%)*	*Number of nuclei per myotube*
Control myoblasts	88 ± 5	15 ± 2
DM1 myoblasts	59 ± 1.4*	3.8 ± 0.5***
DM1 myoblasts, pLXie $(CUG)_{13}$	86 ± 5**	13 ± 1****
DM1 myoblasts, pLXie 5'UTR	59 ± 2ns	3.5 ± 0.4 ns
DM1 myoblasts, pLXie	62 ±2.4 ns	3.5 ± 0.3 ns

Index of fusion was determined in muscle cell cultures grown in a differentiation medium for 5 days. Fusion index was determined by counting the number of nuclei in multinucleated myotubes and it was expressed as percentage of the total number of nuclei. Values represent the mean ± SD of three independent cultures. Statistical analyses were performed by using Student's t-test. *Significantly different from control myoblasts, P=0.03; **Significantly different from DM1 myoblasts, P=0.03; ns=not significantly different from DM1 myoblasts. The mean number of nuclei per myotube was determined in muscle cell cultures after 5 days of differentiation. Values represent the mean ± SD of three different experiments. In total, 70 myotubes were analyzed per culture; ***Significantly different from control myoblasts, P<0.01; ****Significantly different from DM1 myoblasts, P<0.01; ns=not significant from DM1 myoblasts, by Student's t-test.

CHAPTER IV

Hammerhead ribozyme-mediated destruction of nuclear foci in myotonic dystrophy myoblasts

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Running title: Ribozyme-mediated destruction of foci in DM myoblasts

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4.1. Résumé

La dystrophie myotonique de type I (DM1) est causée par une extension instable de CTG dans la partie 3' non-codante du gène de la dystrophica myotonica protéine kinase (DMPK). Les ARNm provenant de l'allèle mutée lient des protéines nucléaires ce qui résulte à la rétention des ARNm mutants de DMPK dans le noyau et la formation de foci qui seraient responsable de la majorité des symptômes de la maladie. Nous avons développé un ribozyme exprimé par un promoteur de tRNAmeti muté capable de se lier à l'ARN de DMPK dans des myoblastes DM1. Pour ce faire, nous avons identifié des sites sur l'ARN de DMPK accessibles pour la liaison du ribozyme. Nous démontrons dans ce rapport que les niveaux d'ARNm normaux de DMPK sont réduits de 50% et ceux des ARNm mutés de 63% dans les myoblastes DM1. De plus, la destruction des ARNm mutés engendre une diminution de l'intensité et du nombre de foci et permet une restauration du niveau d'ARNm de l'isoforme B du récepteur à l'insuline qui est diminué dans les myoblastes DM1. Cette approche à base de ribozyme se démontre donc prometteuse pour le développement d'une thérapie génique à base de ribozymes pour la DM1.

4.2. Abstract

Myotonic dystrophy type 1 (DM1) is caused by an unstable CTG expansion in the 3'untranslated region (3'UTR) of the myotonic dystrophy protein kinase gene (DMPK). Transcripts from this altered gene harbour large CUG expansions that are retained in the nucleus of DM1 cells and form foci. It is believed that the formation of these foci is closely linked to DM1 muscle pathogenesis. Here we investigated the possibility of using a nuclear-retained hammerhead ribozyme expressed from a modified tRNAmeti promoter to target and cleave mutant transcripts of DMPK. Accessible ribozyme target sites were identified in the 3'UTR of the DMPK mRNA and a hammerhead ribozyme was designed to cut the most accessible site. Utilizing this system, we have achieved 50% and 63% reductions respectively of the normal and CUG expanded repeat-containing transcripts. We also observed a significant reduction in the number of DMPK mRNA-containing nuclear foci in human DM1 myoblasts. Reduction of mutant DMPK mRNA and nuclear foci also corroborates with partial restoration of insulin receptor isoform B expression in DM1 myoblasts. These studies demonstrate for the first time intracellular ribozyme-mediated cleavage of nuclear retained mutant DMPK mRNAs, providing a potential gene therapy agent for the treatment of myotonic dystrophy.

KEY WORDS: DMPK, myotonic dystrophy, ribozyme, CUG repeats, gene therapy.

4.3. Introduction

Myotonic Dystrophy is a degenerative neuromuscular disease in which a large repeat expansion in the DNA is responsible for the vast multi-system disorders observed in patients [1]. Myotonic dystrophy type I is caused by a CTG expansion located in the 3'untranslated region of the DMPK gene located on chromosome 19 [2-4]. DMPK is a protein kinase that is thought to be implicated in muscle function and development, but its exact role has yet to be defined [5-7]. Most of the clinical symptoms appear when the expansion at the DM1 locus reaches between 80 to 150 repeats [8,9]. Repeat lengths of 3000 to 4000 are associated with the more severe forms of congenital cases [1,10].

There are three primary mechanisms by which myotonic dystrophy type I may manifest itself [4,11]. First, there is the haplo-insufficiency of the DMPK protein caused by nuclear retention of the mutant transcripts, resulting in a 50% decrease in total DMPK protein in a heterozygous individual [8,12,13]. Mutant DMPK mRNAs containing the pathogenic CUG expansion are transcribed and polyadenylated but accumulate to form foci in the nucleus of DM1 cells where RNA-binding proteins are though to prevent their export from the nucleus [8,13]. An insufficiency of this kinase alone can explain some of the cardiac conduction defects seen in the disease, but cannot explain most of the severe clinical symptoms seen in DM1 patients such as the myotonia and muscle wasting [5,6]. Second, there is transcription downregulation of the neighbouring homeobox gene, Six5 [14-17]. Knock-outs of the murine orthologue of Six5 where shown to develop cataracts, but show no sign of the myopathy seen in DM1 patients [18]. The third potential mechanism leading to some of the symptoms in DM1 can be explained by a toxic gain of function caused by the expansion itself. The first evidence of this was shown in transgenic mice expressing human □-actin mRNA with 250 CUG repeats in the 3'UTR. The mice developed myopathy and myotonia [19]. It was later shown that mice carrying the complete human DM1 locus with 300 CTG repeats also showed histological, electrophysiological and molecular defects consistent with those seen in skeletal muscle of DM1 patients [20].

Current beliefs are that mutant transcripts with large CUG expansions result either in sequestration or elevated levels of critical RNA-binding proteins in the nucleus, involved in various nuclear functions such as splicing [21-23]. Many proteins have been shown to interact with mutant transcripts, but CUGBP1 and the muscleblind family members (MBNL, MBLL and MBXL) are of particular interest [22,24-26]. Elevated levels of CUGBP1 induced by mutant transcripts lead to splicing aberrations of the cardiac troponine T (cTNT) and the insulin receptor [21]. Thus, prevention of mutant DMPK transcript accumulation in the nucleus could possibly alleviate some of the symptoms related to this toxic gain of function caused by the CUG expansions, thereby potentially restoring some of the affected cellular functions.

Since it is certain that mutant transcripts are directly involved in some of the disease mechanisms, and because the DMPK protein does not seem critical for normal muscle development and function [5,6], the mutant DMPK RNA is a suitable target for gene therapy. For gene therapy to be effectively utilized in the treatment of myotonic dystrophy, specific destruction of the mutant transcript is required. A sensible approach should take advantage of the fact that mutant transcripts are retained in the nucleus of DM1 cells thereby allowing preferential targeting of these RNAs. Ribozymes are catalytic RNAs capable of cutting a specific phosphodiester bond on virtually any accessible target RNA with an appropriate cleavage motif (see reference [28] for a review). Because of their small size, RNA polymerase III (Pol III) promoter systems can be used for high levels of ribozyme transcript expression [29] and trans-acting hammerhead ribozymes have been shown to be very effective tools for mediating gene silencing in a wide variety of systems and applications [30,31].

In the present study, we have examined the possibility of using ribozymes to specifically cleave mutant DMPK transcripts by taking advantage of the fact that these mutant RNAs are retained in the nucleus of DM1 cells. We demonstrate that nuclear expression of an anti-DMPK ribozyme results in greater than 60% cleavage of the mutant DMPK message

and also reduction in the number of DMPK nuclear foci. We then show that destruction of the defective RNA and disease-associated foci have a direct effect on partial restoration of the muscle-specific insulin receptor B isoform that is normally downregulated in DM1 myoblasts. Thus, the combination of ribozyme and Pol III promoter system used in this study may potentially be useful for gene therapy of myotonic dystrophy.

4.4. Materials and methods

Primary human muscle cell cultures

Control myoblasts were obtained from a deceased 13 month-old newborn. DM-750 myoblasts were obtained from muscles of a 12 week-old aborted DM fetus with approximately 750 CTG repeats, as verified by southern blot. Skeletal muscle biopsies were approved by Laval University and the CHUL's ethical committees. Myoblasts were grown in MCDB-120 supplemented with 15% heat-inactivated fetal bovine serum, 5 μg/ml insulin, 0,5 mg/ml BSA, 10 ng/ml human hrEGF, 0,39 μg/ml dexamethasone, 50 μg/ml streptomycin and 50 μg/ml penicillin. Differentiation was carried out in DMEM supplemented with 10 μg/ml insulin, 10 μg/ml apo-transferrin, 50 μg/ml streptomycin and 50 μg/ml penicillin.

Accessibility site screening

All ODNs were prepared in the DNA synthesis facility of the Beckman Research Institute (Duarte, CA.). Sequence of antisense ODNs are found in Fig. 1B. DM-750 myoblast cell extract preparations and ODN-RNA mapping with endogenous RNase H were done based on a method previously described [40]: 50 nM of each antisense ODN was incubated for 10 min at 37oC in a mixture containing 20 μl of DM-750 cell extract, 1mM of DDT and 40 U of RNase inhibitor (Invitrogen) in a total volume of 30μl. After incubation, 10 μg of glycogen in a 70 μl volume was added to the mixture and total RNA was extracted using 1 ml of Trizol (Gibco). The RT reaction was performed in a 20 μl volume containing 500 ng of total RNA, 0,4 μM of random hexamers and Stratascript reverse transcriptase (Stratagene). Ten μl of the RT reaction were amplified by PCR using simultaneously 0,1 μM of β-actin primers and 0,8 μM of DMPK 3'UTR primers in a 30 μl volume (Fig. 1B). PCR conditions are as follows: 30 sec. at 94^{o}C, 45 sec. at 62^{o}C and 45 sec. at 72^{o}C for 10 cycles followed by 25 cycles of the following program: 30 sec. at 94^{o}C, 30 sec. at 58^{o}C and

30 sec. at 72°C. The β-actin amplicons serve as loading and internal controls. Fifteen μl of the samples were then loaded on a 1.5% agarose gel and band intensity was measured by densitometry using an AlphaImager imaging system and software (Innotech Corp.).

Vector construction

Wild type (RBZwt) and mutant (RBZmut) hammerhead ribozyme constructs were produced synthetically with the following overlapping primers: forward 5'-CCG CTC GAG CGG AGT CGA ACT G*AT GAG TCC GTG AGG AC- 3'; reverse 5'-GCT CTA GAG CCT AGA ACT GTT TCG TCC TCA CGG AC-3'. The 5' ends of each primer has an endonuclease cleavage site: the forward primer contains an XhoI site and the reverse primer has an XbaI site. The mutant ribozyme (RBZmut) has a G to A transition at position 22 (indicated by an asterisk) in the forward primer. Equal molar ratios (0,5 mM) of the primers were allowed to anneal at 45°C for 5 minutes after a 1-minute denaturing period. Primer extension was done for 2 minutes in standard PCR conditions. Primer extension products were double-digested with XhoI/XbaI and cloned in the SalI/XbaI site immediately 3' of a human tRNAmeti cassette previously described [46] generating the tRNAmet-RBZwt or tRNAmet-RBZmut vectors. These constructs contain an internal tRNA-methionine promoter which is driven by RNA polymerase III and ensures high transcription levels in vivo [46]. The last 10 bases of the mature tRNAmeti have been removed to prevent processing and maturation thus blocking export from the nucleus [50,51]. Immediately 3'of the ribozyme cloning site, there is a stem loop with the following sequence 5'AGCGGACTTCGGTCCGCT-3' which was added for increased resistance to 3' degradation [46]. Adjacent to the stem loop there is a series of five thymidines which serve as a PolIII terminator sequence (see Fig.3A for details).

In-vitro cleavage

A DMPK construct containing part of exon 15, the 3'UTR upstream from the repeats and 100 CTG repeats was prepared from the pRMK-CTG100 vector containing the full length

DMPK cDNA with 100 CTG repeats. The pRMKΔ-100 construct was produced by digesting the pRMK-CTG100 vector using NaeI/XhoI, then blunting and re-ligating. These modifications produce a 806 nt transcript initiated from the T7 promoter in 5'. Run-off transcriptions were done with the tRNAmet-RBZwt or tRNAmet-RBZmut vectors linearized with HindIII and the DMPK substrate was transcribed after the pRMKΔ-100 plasmid was linearized with EcoRI. Transcriptions were done according to the manufacturer's specification included with the T7 RNA polymerase (New England Biolabs). The pRMKΔ-100 plasmid was transcribed in presence of 2μCi/μl [$\tilde{\alpha}$32P] UTP (NEN). In-vitro cleavage of 2 nM of the labeled DMPK substrate was done in a 20μl volume with 50mM Tris-HCl, pH 7.5, 20 mM NaCl, 1mM KCl, and 40 U of RNase inhibitor (Invitrogen). Ribozymes and the labeled substrate were added in the cleavage buffer and heated at 95^{o}C for 2 minutes. Annealing was done by incubation at room temperature for 15 minutes, and then cleavage was initiated by adding $MgCl_2$ and incubating the samples at 37^{o}C. After cleavage, 20 ul of 2X RNA loading buffer (8M Urea, 0.2M EDTA, 15% glycerol, 0.02% (w/v) xylene cyanol and 0.02 % (w/v) bromophenol blue) was added to the samples and 15 μl were loaded on a 4 % polyacrylamide gel with 7M urea. Bands were quantified by densitometry using an AlphaImager imaging system and software (Innotech Corp.). Cleavage percentage was calculated using the following formula: (P1+P2)/(P1+P2+S) X 100% were S is the 806 nt substrate, P1 is the 554 nt 5'cleavage product and P2 the 214 nt 3'cleavage product.

Transfections

Transfections for the Northern blot were done in 60 mm dishes with myoblasts at 70% confluence. Either 7.5 or 15 μg of the ribozyme constructs were co-transfected with either 2.5 or 5 μg of the CMV-REV-eGFP control vector using Lipofectamine 2000 (Life technologies) according to manufacturer's specifications. Transfections were done in differentiating medium in absence of serum. Medium was changed after 16 hours.

DMPK RNA extraction using proteinase K digestion was done as follows

Three ml of fresh lysis buffer was added to confluent myoblasts differentiated for 3 days in a 60mm dish. The lysis buffer contained the following: 500 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1.5 mM $MgCl_2$, 10 mM EDTA, 2% SDS and 0,50 mg/ ml of proteinase K. The cells were harvested after detaching from the dishes and incubated at 45^{o}C for 45 minutes in a shaking water bath. Twenty mg of oligo (dT)-cellulose Type 7 (Amersham Pharmacia Biotech inc.) was added to 2 ml microcentrifuge tubes and washed 3 times with binding buffer (500 mM NaCl, 10 mM Tris-HCl, pH 7.2, EDTA 0.1 mM). After incubation, each cell lysate was separated in two tubes containing oligo (dT)-cellulose. Binding of mRNA to oligo (dT)-cellulose was carried out at room temperature for 30 min on a gentle shaker. Samples were then washed 3 times in wash buffer (250 mM NaCl, 10 mM Tris-HCl, pH 7.2) by pelleting at 4000 x g. for 30 seconds. Oligo (dT)-cellulose was then transferred to small spin columns (QIAGEN) and washed 3 times with 1,5 ml wash buffer by pelleting at 4000 x g. for 10 seconds. Samples were eluted twice with 100 µl of 10 mM Tris-HCl, pH 7.2 and then precipitated with 30 µl of 2M sodium acetate pH 5.2 and 600 µl ethanol in presence of 20 µg of glycogen.

Northern blot

Five hundred ng of poly (A)+ mRNA were loaded on a 1.2% agarose gel with 2.2M formaldehyde and 1 X MOPS buffer. Samples were separated by electrophoresis and then transferred to a Biodyne B nylon membrane (Pall Corp) using a microfiltration apparatus (Pharmacia). Hybridization conditions were previously described [12]. The DMPK probe used was prepared by random priming of 50 ng of the BglII-SacI fragment of the DMPK cDNA (pRMK-CTG100 plasmid). The cyclophilin probe was prepared by random priming of a 682 bp PCR product amplified from cyclophilin cDNA. Densitometry analysis was done on the original autoradiograms with an AlphaImager imaging system and software (Innotech Corp.).

In situ hybridization

Myoblasts were grown on glass coverslips inserted in a 24 well plate. Cells were transfected at 70% confluence as described earlier with either the wild type or mutant ribozyme construct and the CMV-REV-eGFP reporter plasmid. The REV-eGFP fusion protein was previously shown to accumulate in the nucleoli of cells [30]. Cells were then induced to differentiate and fixed on the third day. Fixation, permeabilization and hybridization conditions were done according to the Singer lab protocols found here: http://singerlab.aecom.yu.edu/protocols. The PNA Cy3-$(CAG)_5$ probe used was kindly provided by Dr. T.L. Taneja (Boston Probes). The images were collected on an Axiophot fluorescence microscope (Zeiss) and PVC100C digital camera (Pixera).

RT PCR

Normal and DM-750 myoblasts were grown in 100mm plates and co-transfected at 70% confluence with the CMV-REV-eGFP reporter plasmid and either tRNA-RBZwt or tRNA-RBZmut constructs. Cells were then sorted the next day for eGFP expression and plated at 90% confluence in a 24-well plate with differentiating medium. Myoblasts were allowed to differentiate for 4 days and total RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer's specifications. First strand cDNA synthesis and PCR amplifications of the insulin receptor A and B isoform cDNAs were done using identical primer sequences and assay conditions as previously reported [21]. PCR amplification resulted in a 131 bp amplicon for isoform A and a 167 bp amplicon for isoform B. PCR products were resolved on a 2.5% agarose gel and densitometry analysis was done using an AlphaImager imaging system and software (Innotech Corp.). PCR assays were performed in the linear range of the amplifications for both IR-A and IR-B amplicons, which is between 20 and 35 cycles in our experimental conditions.

4.5. Results

Identification of accessible ribozyme target sites

The most critical step when designing a ribozyme approach for targeted transcript destruction is the choice of a ribozyme cleavage site. Although hammerhead ribozymes can cleave any RNA with the NUH consensus sequence, where N can be any ribonucleotide and H can be A, C or U, there are several other crucial factors to consider when determining accessibility [32,33]. Two of these factors are the formation of predictable secondary structures and the presence of RNA-binding proteins associated with the target transcript. There are several computer based RNA folding programs that predict local secondary structures, but to date, these have not proven to be routinely useful for predicting ribozyme accessible sites in vivo. The folding of RNAs in the presence of RNA chaperoning proteins may not allow accurate predictions of ΔG, which is the parameter used for predicting stability of secondary structures [34]. In the case of DMPK mRNA, it is well known that the expanded CUG repeats form very stable structures once the number of triplet repeats approaches 30. With larger repeats, DMPK RNA structures are discernible using electron micrographs [35,36]. Thus it is intuitive that difficulties will arise when attempting to target such structures with antisense or ribozyme reagents. Moreover, it has been shown that many RNA-binding proteins interact with CUG repeats such as CUG-BP1, the muscleblind family members and PKR, a doubled-stranded RNA binding protein [24,26,37]. A final consideration in determining the right target site is splicing variants. In the case of the DMPK mRNA, 7 different isoforms have been identified with the possibility that others may exist as well [38,39]. In order to circumvent the above stated limitations for a ribozyme cleavage site, we have thus chosen the 3' untranslated region between the UAG termination codon and the beginning of the repeat expansion for possible ribozyme cleavage sites (Fig. 1A). This region is conserved among the 6 known DMPK isoforms that contain the expansions [38,39]. Within this region we utilized an oligonucleotide-based screening assay to analyze 5 potential hammerhead ribozyme cleavage sites for accessibility to base pairing (Fig. 1B).

We used the antisense oligodeoxyribonucleotide (ODN) based assay in cell extracts to determine the accessible sites for antisense and ribozyme pairing [40-42]. Briefly, we incubated differentiated DM-750 myoblast extracts with antisense ODNs targeted to the regions of DMPK shown in figure 1A. If the ODN efficiently pairs with the target sequence, a substrate for endogenous RNase H in the extracts is created and the RNA is cleaved in the region of oligo pairing. RT-PCR reactions are then carried out on the target transcript and on a control transcript to quantitate the extent of cleavage. Figure 2A shows the results of our base-pairing accessibility experiments. These data show that 3 target sites out of the 5 tested are accessible to base-pairing with the ODNs under the conditions utilized. The RT-PCR experiments were multiplexed with both DMPK and β̃actin primer sets to enable normalization between samples. Normalized quantifications of the amplicons show that target site #1 was reduced by 94% and site #2 by 82% (Fig. 2B). The least accessible site was #5, which lies the closest to the CUG expansion track.

Ribozyme construction and promoter selection

An important step in ribozyme design is the choice of an appropriate expression system that facilitates co-localization of the ribozyme and target [43]. Co-localizing ribozymes in the same cellular compartment as the target greatly improves ribozyme function [41,44]. In myotonic dystrophy, mutant DMPK mRNAs are retained in the nucleus. We therefore chose an expression system that results in nuclear retention of the ribozyme transcripts, and this is based upon a structurally altered tRNAmeti promoter system preventing RNA processing and export [45-47]. A wild-type and a mutant hammerhead ribozyme targeted to site #1 were designed and cloned in the expression cassette 3' of the tRNAmeti coding region and 5' to a stem loop configuration that is intended to reduce 3' to 5' degradation of the transcripts (Fig. 3A). The mutant ribozyme has a G to A transition in the catalytic core that completely abolishes its cleavage activity, but still allows antisense pairing. The complete structure and sequence of the hammerhead ribozyme used in our experiments is depicted in Fig. 3B.

In vitro cleavage of DMPK mRNA

Wild type, mutant and non-sense ribozymes were transcribed in vitro by initiation from the T7 promoter of our constructs. A transcript of 806 nt containing most of exon 15 and the first 535 nt of the 3'UTR with 100 CUG repeats was initiated from the vector's T7 promoter. Figure 4 depicts a series of ribozyme reactions using this highly structured substrate in vitro. In figure 4A, it can be seen that cleavage efficiency is dependent upon the ribozyme to target ratio, 45% cleavage of the target was only achieved at a ratio of ribozyme to substrate of 150:1 over the course of 1 hour using 10 mM $MgCl_2$. The need for such a huge excess of ribozyme to substrate reflects the relative inaccessibility of the substrate in the absence of cellular chaperoning proteins. When the $MgCl_2$ concentration was increased to 20mM, there was an enhancement of cleavage as seen in Fig. 4B. Figure 4C illustrates a time course utilizing the 150 fold excess of ribozyme with 20mM $MgCl_2$. Under these conditions, there was 64% cleavage of the DMPK substrate in 2 hours. No cleavage activity was measured with either the mutant ribozyme or an unrelated ribozyme (Fig. 4D).

The hammerhead ribozyme preferentially targets the mutant DMPK transcript

To investigate the intracellular activity of our ribozyme constructs, we transfected the wild type and mutant tRNAmet constructs into DM-750 myoblasts. The ribozyme vectors were co-transfected with a reporter gene in a ratio of 3:1 to estimate transfection efficiency of the ribozymes. The control vector expresses a REV-eGFP fusion protein that localizes in the nucleoli of cells (Michienzi and Rossi, unpublished observations). Transfections were carried out using differentiation medium that does not contain serum. This allows for higher transfection efficiencies as well as initiating differentiation at the moment of transfection. Our objective was to ask whether the functional ribozyme could cleave the DMPK mRNA during myoblast differentiation. After 3 days of differentiation, the myoblasts were harvested and mRNA was extracted using a proteinase K extraction

method (Langlois et al, manuscript submitted). This is the only method that we have found that allows complete extraction of mutant DMPK mRNAs that are sequestered in nuclear foci. A Northern blot using mRNA from transiently transfected DM-750 myoblasts probed with a 32P-labelled DMPK cDNA fragment shows equal expression of both the normal 2.8kb DMPK transcript and the disease-associated 5.1 kb mutant transcript with 750 CUG repeats (Fig. 5., lane 1). DMPK expression is globally diminished in cells transfected with the wild type ribozyme construct, but with a preferential decrease of mutant DMPK (Fig. 5., lane 3) in comparison to cells transfected with the non-functional mutant ribozyme construct (Fig.5.,lanes 4 and 5). Transient tranfections were carried out using either 10 or 20 μg of total DNA. The best transfection conditions were obtained using a total of 20μg of DNA, resulting in an estimated 60% transfection efficiency of the ribozyme. Under these conditions we observed 63% and 50% reductions of the mutant and normal DMPK transcripts respectively. Transfection using 10 ug of DNA yielded approximately 26% efficiency which resulted in 25% reduction of mutant and 19% reduction of normal DMPK mRNAs. Results presented in figure 5 represent one of 4 experiments giving similar results. Normalization was done using cyclophilin expression and values were calculated based upon DMPK message in cells transfected with mutant ribozyme DNA.

Destruction of nuclear speckles by ribozyme

In order to validate the Northern blotting results, we investigated the extent of nuclear foci reduction in cells treated with the functional versus the mutant ribozyme. We transiently co-transfected DM-750 myoblasts with either the tRNAmet-RBZwt or tRNAmet-RBZmut constructs and with the CMV-REV-eGFP control plasmid. After a 3-day differentiation period, an in situ hybridization was performed using a Cy3-labeled PNA-$(CAG)_5$ probe. Foci in two hundred eGFP-positive myoblasts co-transfected with either tRNA-RBZwt or tRNA-RBZmut constructs were numbered. Our results demonstrate that there is a reduction in the number and intensity of nuclear foci in wild type ribozyme-transfected DM-750 cells compared to mutant ribozyme-transfected cells (Table 1). Approximately 88% of all DM-750 myoblasts contain between 6 and 15 foci, this number dropped to between 0 and 5 foci

for 90 % of DM-750 myoblasts expressing the wild type ribozyme. Also, foci were absent from approximately 20% of all eGFP-positive myoblasts transfected with tRNA-RBZwt. These data clearly demonstrate that the DMPK mutant message was cleaved by the wild type ribozyme and this resulted in a substantial reduction of mutant DMPK mRNA-associated nuclear foci. Figure 6 shows a sample field of the in situ hybridization with DM-750 myoblasts co-transfected with CMV-REV-eGFP and either tRNA-RBZmut or tRNA-RBZwt. In the left panels, we see that nuclear speckles form in the nucleus of all cells either transfected or not. DAPI staining gives a clear indication of the nucleus boundary. Panels on the right show that foci are absent in the eGFP-positive cell, but are numerous in eGFP-negative cells next to it that most likely do not express the ribozymes.

Insulin receptor splicing

Many associations have been made in the past few years between the expression of mutant DMPK mRNA with large CUG expansions and cellular and molecular defects observed in DM1 cells and patients. Some of these phenotypes relate to defective splicing in several mRNAs which include cardiac troponine T [22], chloride channel 1 [48], tau [23] and the insulin receptor [21]. Having succeeded in partially destroying mutant DMPK mRNA and reducing the number of nuclear foci, we now investigated whether normal splicing of the insulin receptor could be restored. Normal skeletal muscles express predominantly the type B insulin receptor isoform which has a higher capacity for signalling, but in DM1 an alternative splicing defect causes the low signalling isoform A to be expressed in majority [21]. Inclusion of exon 11 in the type B isoform is responsible for this receptor's higher signalling capability required in insulin-responsive tissues [21]. To determine the effect of our wild type ribozyme on insulin receptor isoform expression, we co-transfected normal and DM-750 myoblasts with the CMV-REV-eGFP reporter and with either the tRNA-RBZmut or tRNA-RBZwt constructs. Cells were sorted for eGFP expression 24 hours post-transfection and plated to confluence in differentiating medium. Total RNA was extracted after 4 days and an RT-PCR was performed. Figure 7 shows that normal myoblasts transfected with the ribozymes express predominantly the B isoforms. DM-750 myoblasts express dominantly the A isoform but restoration of isoform B expression is obtained when

the tRNA-RBZwt ribozyme is transfected. The average mean of B to A ratios for two combined experiments were of 1.25 ± 0,21 (mean ± S.E., n=2) and 1.15 ± 0,31 (mean ± S.E., n=2) for normal myoblasts expressing the mutant and wild type ribozymes and of 0,44 ± 0,12 (mean ± S.E., n=2) and 0,85 ± 0,14 (mean ± S.E., n=2) for DM-750 expressing the mutant and wild type ribozymes respectively. In sum, restoration of insulin receptor isoform B in DM-750 myoblasts transfected with the tRNA-RBZwt construct increased from 33% to 65% of the values seen in the normal myoblast controls. These results clearly unveil the link between the expression of mutant DMPK mRNA with large CUG repeats and some of the biological phenotypes seen in DM cells and tissues.

4.6. Discussion

Much evidence suggests that retention of the mutant DMPK RNA and foci formation is strongly involved in DM1 muscle pathogenesis. We have demonstrated that a hammerhead ribozyme targeted to a conserved region of the DMPK mRNA is capable of blocking the formation of foci in DM-750 myoblasts by destroying the DMPK mRNA. In order to achieve a functional ribozyme against this target, several important issues were considered. The first issue was to target all known splicing variants of the DMPK transcripts. Many protein isoforms have been identified and all of them containing the CUG track possess the target site selected. Secondly, we needed to target DMPK mRNA from various donors and cell lines. No polymorphisms have been identified in the target region. There is no other way to specifically target mutant DMPK mRNAs with a ribozyme other than by taking advantage of the fact that the CUG expanded repeat containing transcripts are retained in the nucleus of DM1 cells. Although complete destruction of all DMPK transcripts with concomitant introduction of a resistant cDNA capable of expressing DMPK is a potential alternative approach, the nuclear co-localization strategy appears to be effective and more simple.

In the in vitro cleavage reactions we observed only 64 % cleavage of our substrate with a 150 fold molar excess of ribozyme over substrate RNA. This is most likely due to the strong secondary and tertiary structures in the DMPK substrate we used. These structures may result in a sub-population of targets that are inaccessible to ribozyme binding in the absence of cellular proteins. Interestingly, this same site is readily cleaved by RNase H in cell extracts. Complete cleavage of a target sequence by hammerhead ribozymes in vitro is seldom obtained with any substrate. Another pertinent point is the length of the ribozyme hybridizing arms which are 12 nucleotides in helix III and 9 nucleotides in helix I. The 21 bases of complementary sequence allow for more efficient on-rates, but impede product dissociation and hence ribozyme turnover in vitro.

For intracellular testing, we expressed the ribozyme from a Pol III promoter that results in nuclear localized transcripts [47]. Some of the main advantages of using a Pol III promoter like tRNAmeti versus Pol II promoters are the high transcription rates of such promoter systems and also because the promoter sequence is transcribed, thereby perhaps conferring some nuclease resistance to the ribozyme. Our Northern hybridization results probing for the DMPK mRNA targets demonstrated that both mutant (nuclear retained) and wild type (cytoplasmic) transcripts were destroyed, although the mutant transcripts were destroyed to a greater extent, perhaps owing to their nuclear localization. Because of the specificity and activity of our ribozyme, it is most likely that the normal transcripts were cleaved during transcription or just prior to export from the nucleus. Since the nuclear compartment in which the DMPK mutant transcripts accumulate (foci) has not yet been characterized, an expression system favouring accumulation directly in the DM1 foci is not yet conceivable. However, reduction of the number of disease-associated foci in the nucleus of DM-750 myoblasts transfected with the wild type ribozyme construct shows that these nuclear structures are reachable and sensitive to degradation. The link between nuclear accumulation of the defective RNAs and disease manifestations such as insulin receptor B isoform downregulation is another direct example of the alterations caused by the pathogenic foci. In our assays, B isoform expression was not optimal because the length of the differentiation was limited to 4 days because of the transient expression of our transgenes. An optimal differentiation period for myoblasts varies between 5 to 10 days depending on culture conditions and parameters needed to be studied.

Concerns and issues have been raised about using ribozymes in vivo, mainly regarding the relative efficiency of these target specific cleavage agents in an intracellular milieu. Nevertheless, recent studies have demonstrated that when an accessible target site has been selected along with co-localization of the ribozyme and target RNA in the cell, ribozyme function is greatly enhanced. One report has demonstrated near complete target cleavage when the ribozyme and target were co-localized in the nucleoli of yeast cells [44]. In our case, we now have a good ribozyme target that will allow us to better test variables in

ribozyme expression and the roles of sequences flanking the ribozyme on catalytic function. Because transfection efficiencies are generally poor and very variable in primary culture myoblasts, and also because growing cells for many days post-transfection is necessary to assess phenotypes of differentiated DM cells, we are currently exploring the use of a lentivirus-based vector system to deliver our ribozyme expression system. This system will allow us to work with homogenous cultures expressing wild type or mutant ribozymes. Our main objectives will be to investigate if destruction of the nuclear foci and mutant RNAs will allow restoration of other cellular and molecular DM1 phenotypes such as glucose uptake, myoblast fusion and differentiation and the splicing of other defective mRNAs. These tools will be essential to answer some long standing questions about the effects of foci formation in DM myoblasts.

The complex interaction of cellular alterations observed in DM1 cells have provided a wealth of questions over the more than 10 years following the initial identification of the expansion in the DM1 locus on chromosome 19 [3]. Important questions are what cellular defects are associated with the presence of the CUG expansions in the RNA, and what types of chromatin defects are caused by the CTG? By developing a ribozyme capable of the preferential targeting of mutant DMPK transcripts associated with DM1, we provide first and foremost a tool to address these issues. This tool can also give insight into the functional consequences of the nuclear sequestration of the mutant DMPK messages. The ribozyme approach for targeting and destroying mutant DMPK RNAs will hopefully open the path to develop a potential reagent for gene therapy treatment for DM1 as well and also for DM2, where retention of an RNA with expanded four base repeats is also thought to be involved in the pathogenesis of the disease [49].

4.7. Acknowledgements

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4.9. Figure legends

Figure 1. Selection of candidate target sites in the 3' untranslated region of the DMPK mRNA. (A) Schematic representation of antisense ODN target sites in the 3'UTR of DMPK mRNA and primer annealing sites. Normal DMPK mRNA is approximately 2.8 kb in length. Five target sites were selected between the termination codon (UGA) and the beginning of the repeat expansion. (B) ODN and primer sequences used for accessibility site determination.

Figure 2. Identification of accessible ribozyme cleavage sites. (A) Agarose gel showing accessible sites. Whole cell extracts were prepared from differentiated DM1 myoblasts with 750 repeats. Antisense ODNs were added for 5 min. and then total RNA was isolated from the extracts. RT-PCR was then performed using DMPK and β-actin primers. Amplification of a band at 235 bp indicates an inaccessible target site in 3'UTR. The β-actin amplicon at 348 bp serves as an internal loading control. (B) Histogram showing quantification by densitometry of band amplification in three unrelated experiments (n=3).

Figure 3. DNA construct used for ribozyme expression. (A) tRNAmet-ribozyme expression cassette. Transcripts can either be produced in vitro by T7 RNA polymerase or by RNA polymerase III using the internal tRNA-methionine promoter when transfected into myoblasts. There is a sequence coding for a stem loop in 3' of the cassette that confers resistance to 3' to 5' degradation of the RNA. Deletion of the final 10 nucleotides of the mature tRNAmeti transcript prevent its maturation and export from the nucleus [45, 46]. Transcription by Pol III is terminated by a stretch of 5 thymidines. (B) Hammerhead ribozyme sequence and structure with it's DMPK target. The mRNA cleavage site is located after the GUC consensus on the DMPK mRNA. The ribozymes's catalytic activity

is dependent on the presence of the guanosine indicated by an asterisk. The mutant ribozyme used in our assays was generated by replacing the G by an A.

Figure 4. In vitro cleavage assays of a transcribed DMPK substrate. A 32P-UTP-labelled DMPK RNA substrate was produced by in vitro transcription of the pRMKΔ-100 vector which contains a NarI/XhoI deletion of the pRMK-CTG100 vector generating a 806 nt transcript with 100 CUG repeats. Ribozymes were produced by in vitro transcription of the tRNAmet-RBZ construct digested with HindIII. All transcriptions were initiated by a T7 promoter. Ribozyme cleavage of the substrate generates fragments of 214 nt and 554 nt. (A) Target/RBZ ratio optimization. Two nM of labelled substrate was incubated for 1 hour with various concentrations of ribozymes in presence of 10 mM $MgCl_2$. (B) $MgCl_2$ requirement optimization. Two nM of labelled substrate was incubated for 1 hour in presence of 100 nM of ribozyme and varying concentrations of $MgCl_2$. (C) Cleavage time-course. Two nM of labelled substrate were incubated for various periods in presence of 100 nM of ribozyme and 20mM $MgCl_2$. (D) Two nM of labelled substrate were incubated for various periods in presence of 100 nM of mutant and non-sense ribozymes in presence of 20mM MgCl2.

Figure 5. Ribozyme-mediated cleavage of DMPK mRNA by transient transfection of tRNAmet-RBZ constructs in DM-750 myoblasts. DM-750 myoblasts were grown in a 60 mm dish and transfected with either 10 or 20 μg of total DNA containing wild-type or mutant tRNAmet-RBZ constructs and the CMV-REV-eGFP construct. The transfection ratio of ribozyme to reporter was 3 to 1. Cells were then differentiated for 3 days and mRNA was extracted by proteinase-K/polyA(+) isolation. Normal DMPK transcripts (less than 20 CUG repeats) have a length of 2.8 kb and mutant transcripts from DM myoblasts with 750 CUG repeats generate a band at 5.1 kb. One representative experiment of four is shown here.

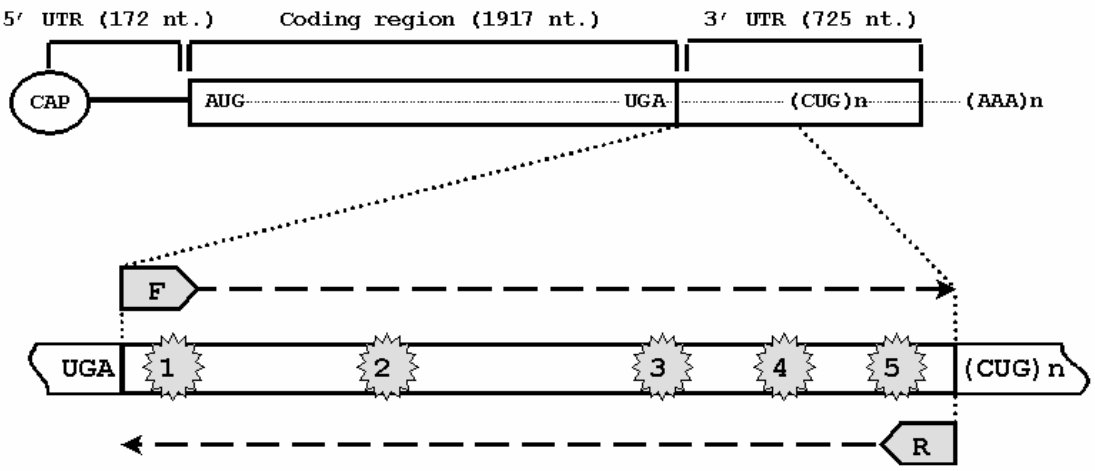
Figure 6. Destruction of nuclear speckles in ribozyme-transfected DM-750 myoblasts. Normal and DM-750 myoblasts were co-transfected with either wild type or mutant ribozyme constructs and the CMV-REV-eGFP vector. The transfection ratio of ribozyme to reporter was 3 to 1. The cells were then differentiated for 3 days. After fixation and permeabilization cells were hybridized with a PNA Cy3-$(CAG)_5$ probe. Red speckles show sequestered mutant DMPK transcripts, the REV-eGFP fusion protein is depicted in green within the nucleoli of the myoblasts and in blue is a DAPI staining of the nucleus.

Figure 7. Restoration of insulin receptor splicing. Normal differentiated myoblasts predominantly express the B isoform of the insulin receptor. In DM1 myoblasts, the insulin receptor A isoform is predominantly expressed. RT-PCR analysis of insulin receptor splicing in normal and DM-750 eGFP-positive myoblasts transfected with either tRNA-RBZmut or tRNA-RBZ-wt constructs. Myoblasts were allowed to differentiate for 4 days before total RNA was extracted. Amplification products are of 131bp for the A isoform and 167bp for the B isoform of the insulin receptor using primers flanking the boundaries between exons 10 and 12 [21]. One representative experiment of two is shown.

4.10. Figures

FIGURE 1

A



B

Target sequences (5′ - 3′)	PCR primers (5′ - 3′)*
1: CCU AGA ACU **GUC** UUC GAC UCC	F: TGA ACC CTA GAA CTG TCT
2: CCA GCU CCA **GUC** CUG UGA UCC	R: GCA GCA GCA TTC CCG GCT
3: AGG GGC CGG **GUC** CGC GGC CGG	*Generates a 235 bp amplicon
4: GCU CGA AGG **GUC** CUU GUA GCC	
5: CAG CCG GGA **AUG** CUG CUG CUG	

FIGURE 2

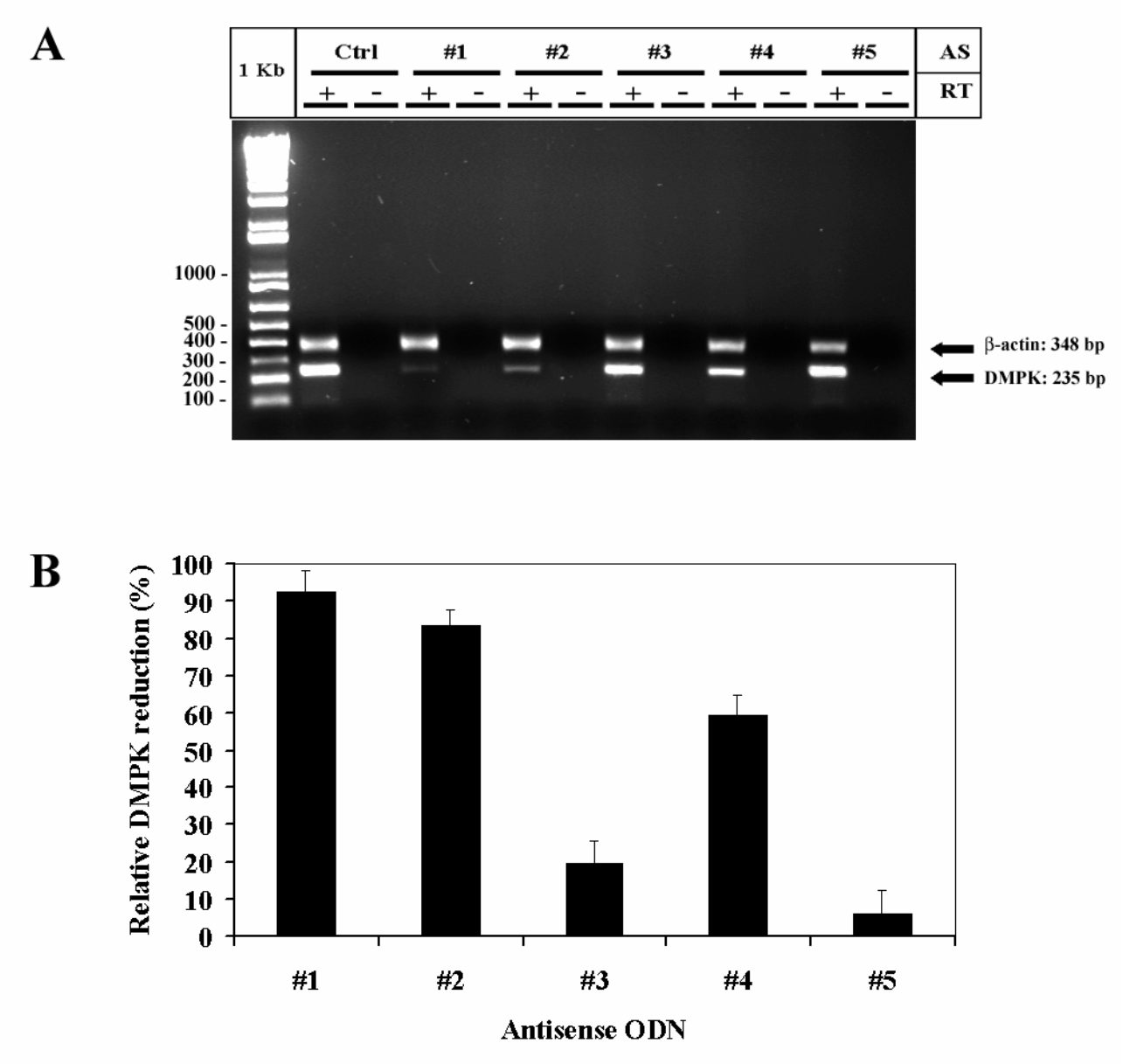

A
1 Kb
Ctrl
#1
#2
#3
#4
#5
AS
RT
+ - + - + - + - + - + -
1000 -
500 -
400 -
300 -
200 -
100 -
β-actin: 348 bp
DMPK: 235 bp
B
Relative DMPK reduction (%)
100
90
80
70
60
50
40
30
20
10
0
#1
#2
#3
#4
#5
Antisense ODN

FIGURE 3

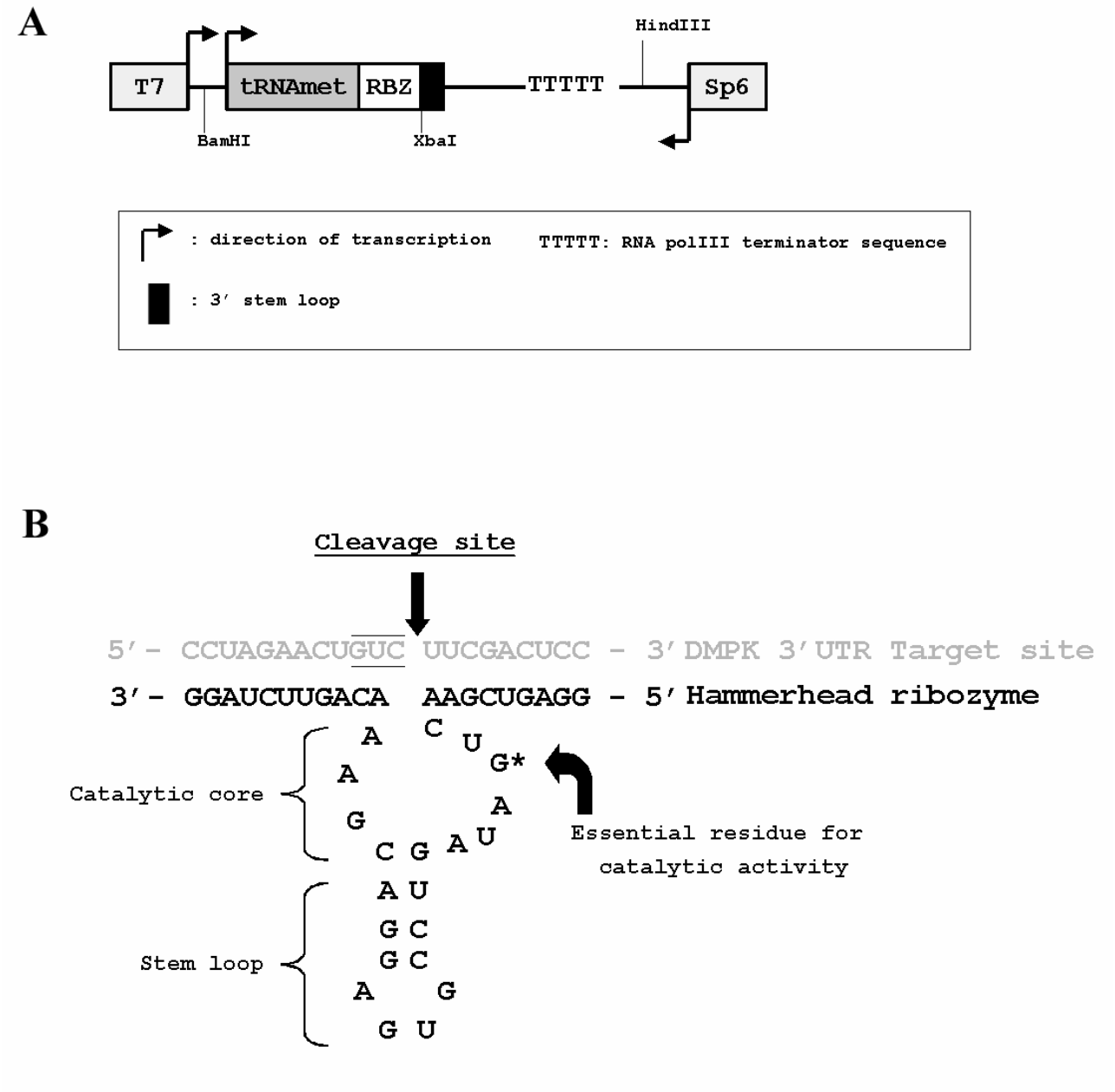

A
T7
tRNAmet
RBZ
TTTTT
Sp6
HindIII
BamHI
XbaI
: direction of transcription
TTTTT: RNA polIII terminator sequence
: 3′ stem loop
B
Cleavage site
5′- CCUAGAACUGUC UUCGACUCC - 3′ DMPK 3′UTR Target site
3′- GGAUCUUGACA AAGCUGAGG - 5′ Hammerhead ribozyme
Catalytic core
Stem loop
Essential residue for
catalytic activity

FIGURE 4

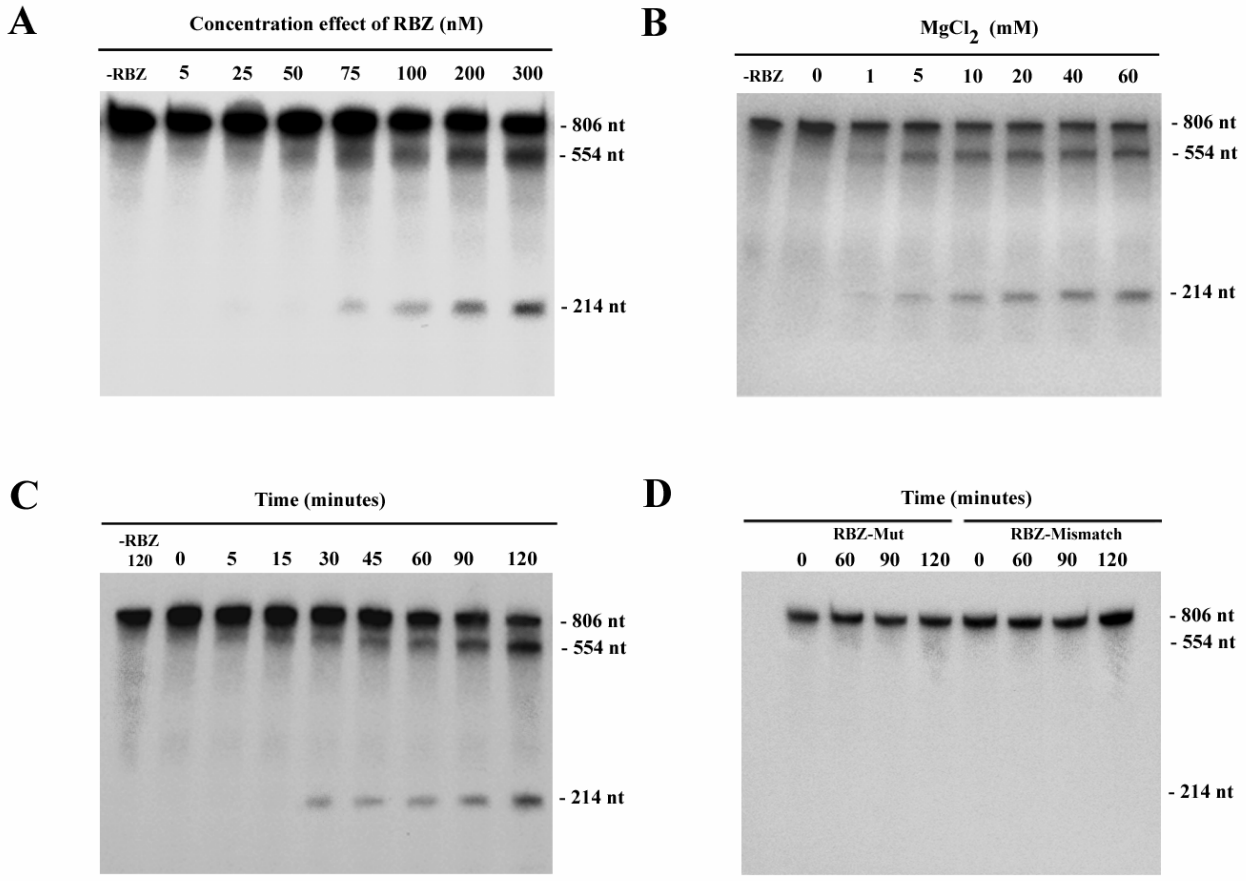

A
Concentration effect of RBZ (nM)
-RBZ 5 25 50 75 100 200 300
- 806 nt
- 554 nt
- 214 nt
B
MgCl2 (mM)
-RBZ 0 1 5 10 20 40 60
- 806 nt
- 554 nt
- 214 nt
C
Time (minutes)
-RBZ 120 0 5 15 30 45 60 90 120
- 806 nt
- 554 nt
- 214 nt
D
Time (minutes)
RBZ-Mut
RBZ-Mismatch
0 60 90 120 0 60 90 120
- 806 nt
- 554 nt
- 214 nt

FIGURE 5

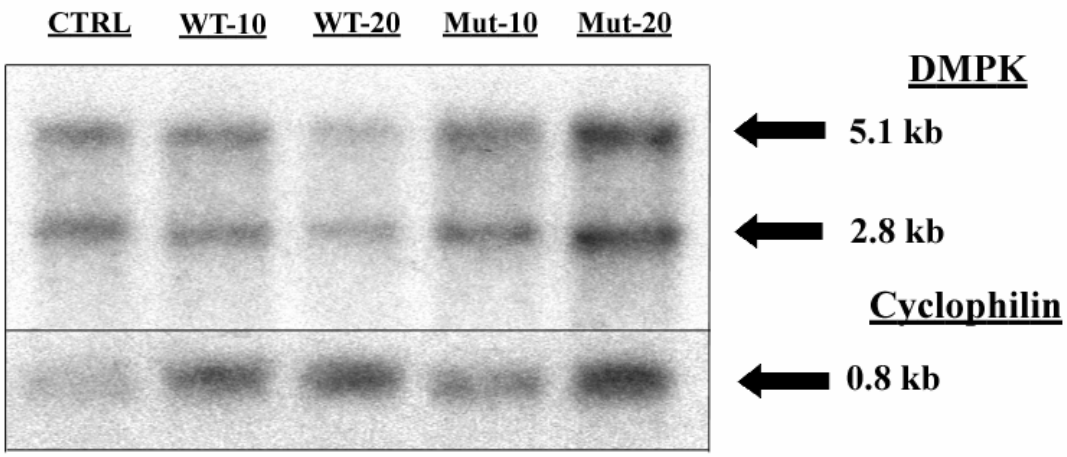


FIGURE 6

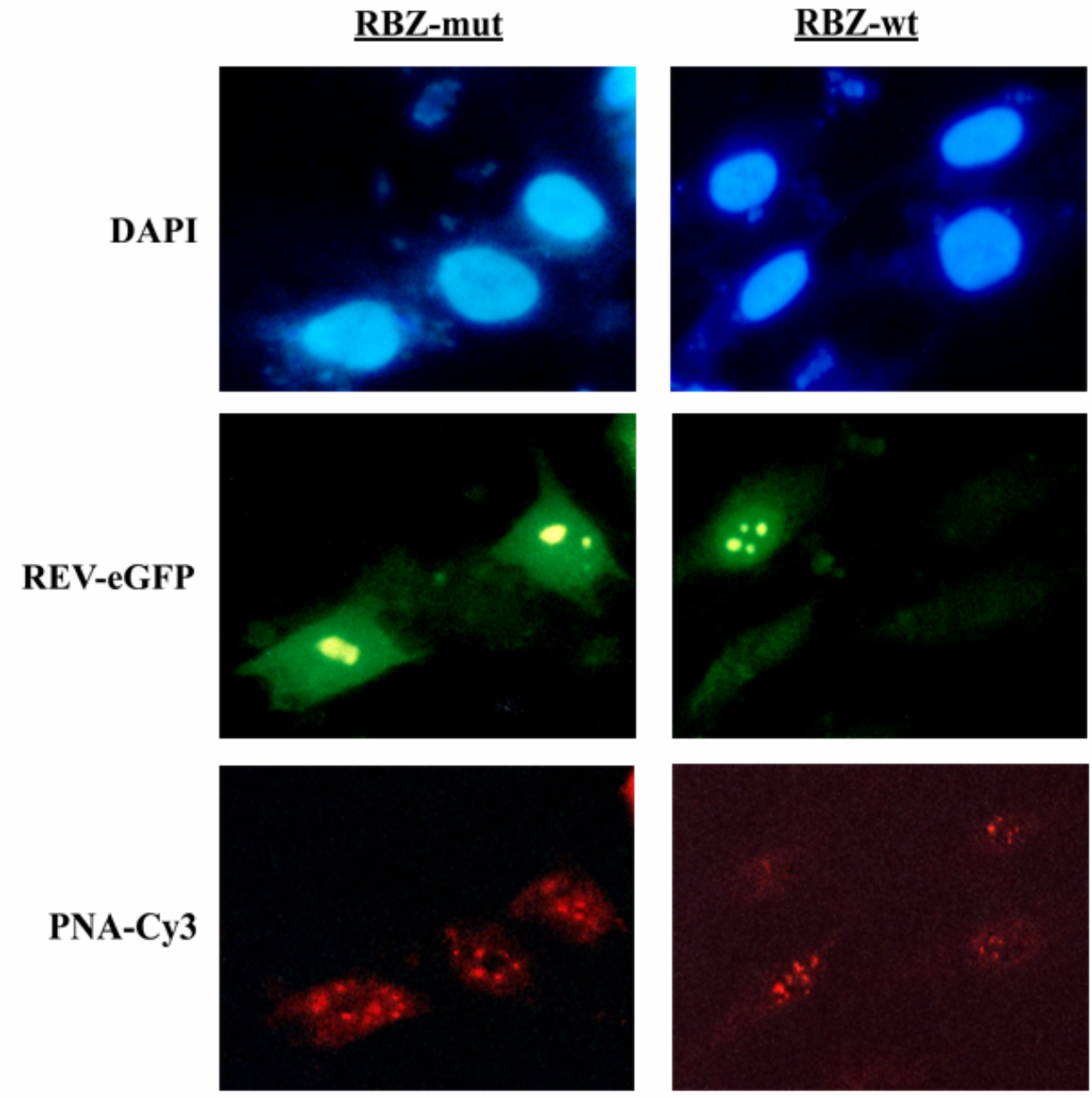
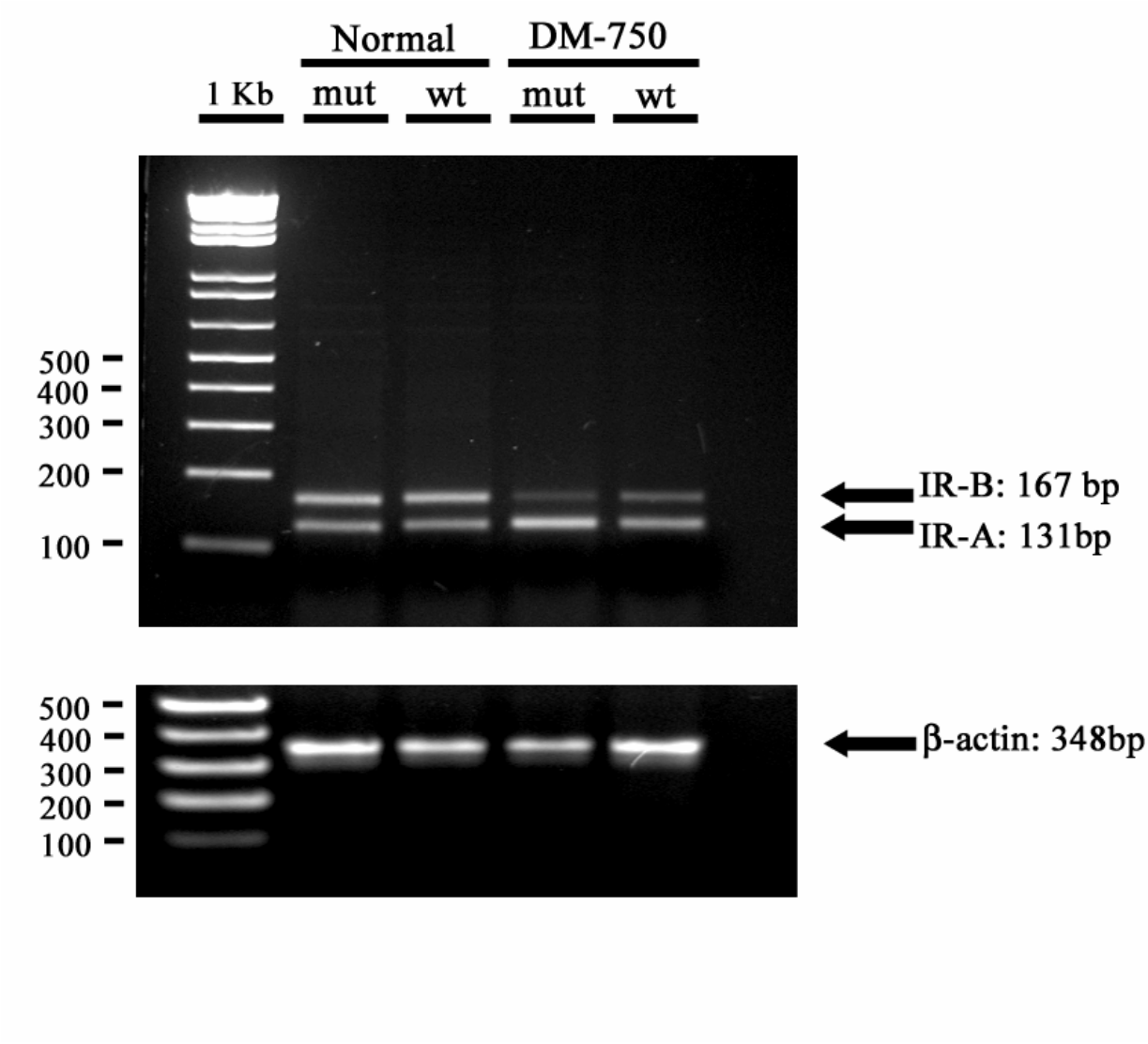


FIGURE 7


Normal
DM-750
1 Kb
mut
wt
mut
wt
500
400
300
200
100
IR-B: 167 bp
IR-A: 131bp
500
400
300
200
100
β-actin: 348bp

4.11. Tables

Table 1. Number of defined mutant DMPK foci visible in the nucleus of ribozyme-transfected DM-750 myoblasts

NUMBER OF FOCI PER NUCLEUS	RBZMUT (%)	RBZWT (%)
0	0.0	19,5
1-5	7.5	70,5
6-10	48.5	9.0
11-15	39.5	1.0
16-20	4.5	0.0

The total number of visible foci were counted in GFP-positive DM-750 myoblasts co-transfected with either the mutant (RBZmut) or wild type (RBZwt) tRNAmeti constructs. This table expresses the percentage of cells in each interval of foci number for both RBZmut and RBZwt. The mean foci number in DM-750 myoblasts transfected with the tRNA-RBZmut and tRNA-RBZwt constructs is 10 ±0,22 (mean ± S.D., n = 200) and 2,5 ± 0,15 (mean ± S.D., n = 200) respectively. These results are the sum of two independent experiments using both tRNA-RBZmut and tRNA-RBZwt.

CHAPTER V

Downregulation of an endogenous nuclear-retained mutant DMPK mRNA by short hairpin RNAs in human primary myoblasts

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Summary: Nuclear RNAi (-like) phenomenon

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5.1. Résumé

Les petits ARNs double-brins (siRNA) s'avèrent être de puissants outils pour diminuer l'expression de gènes spécifiques. Les siRNA sont des duplexes d'ARN d'une longueur de 21 à 23 paires de bases ayant 2 à 3 nucléotides non-appariés à chaque extrémité. Lorsque ces siRNA sont complémentaires à un ARNm cible, nous observons une dégradation de cette cible. Ce mécanisme se nomme l'interférence par l'ARN (RNAi). Il est connu que le siRNA se lie à un complexe nommé RISC (RNA-induced silencing complex) dans lequel les deux brins sont dissociés afin de servir d'appât pour l'ARNm cible complémentaire afin qu'il soit dégradé par le complexe. Plusieurs équipes de recherche soutiennent que ce mécanisme s'effectue uniquement dans le cytoplasme de la cellule, nous présentons toutefois des preuves qu'il existe un mécanisme nucléaire permettant la dégradation des ARNs cibles. Ces résultats suggèrent qu'il existerait une composante nucléaire du RNAi servant probablement au maintien de l'intégrité du génome ou peut-être même à une surveillance immunologique.

5.2. Abstract

RNA interference (RNAi) in mammalian cells is a phenomenon by which a short double-stranded RNA (dsRNA) directs enzyme-dependent cleavage of a specific target RNA. Although it is known that RNAi requires incorporation of one of the short RNA strands into an RNA-induced silencing complex (RISC), the exact cleavage mechanism remains unknown. Most reports have established a cytoplasmic localization and function for the RISC, here we report the cleavage of a nuclear-retained mutant DMPK mRNA by short hairpin RNAs (shRNAs) in human primary myoblasts. Our evidence supports a nuclear RNAi (-like) pathway that does not depend on the cytoplasmic RISC.

KEY WORDS: DMPK, myotonic dystrophy, mRNA, nucleus, shRNA, RNAi

5.3. Report

Short RNA duplexes of 21-23 nucleotides called small interfering RNAs (siRNAs) induce the specific cleavage of target RNAs in mammalian cells. The involvement of small RNAs in controlling gene expression has been termed RNA interference (RNAi) (*1, 2*). RNAi has emerged in recent years as one of the most powerful mechanisms to achieve post-transcriptional gene silencing (PTGS) *in vitro* as well as *in vivo* (3). However, the biological role of RNAi remains unclear. Despite the fact that RNAi has often been used as a tool for downregulation of specific genes in mammalian cells, its mechanism has not been obvious.

Since most protein components of RNAi assemble into the RNAi machinery in the cytoplasm, RNAi has long been viewed as a cytoplasmic mechanism (*4, 5*). Double-strand RNAs (dsRNAs) associate with cytoplasmic Dicer and several members of the ARGONAUTE protein family (*6, 7*). In addition to cytoplasmic pathways, recent work has suggested that RNAi in non-plant systems may also operate by preventing the production of new mRNA by epigenetic silencing of gene expression in the nucleus (8). Deletion of RNAi components in fission yeast has resulted in transcriptional derepression through histone methylation in the nucleus (*9*). This distinct transcriptional gene silencing (TGS) occurs only when the sequence identity occurs within the promoter in contrast to PTGS which occurs only when there is sequence identity within the coding sequence (8).

Here we report the existence of a nuclear mechanism capable of PTGS driven by shRNAs. Myotonic dystrophy type 1 (DM1) is a neurodegenerative disorder caused by a large unstable CTG expansion in the 3'UTR of the DMPK (DM protein kinase) gene (*10*). Mutant transcripts (mt) harbouring the CUG repeats are fully transcribed and polyadenylated, but are not exported from the nucleus (*11*). Complete nuclear retention of mt DMPK mRNAs with large CUG repeats is believed to be one of the most important

sources of molecular disruptions observed in DM1 (*12*). Here we exploit the natural property of nuclear-retained mt DMPK mRNAs to demonstrate that a siRNA-directed cleavage mechanism occurs in the nucleus of the cell.

To demonstrate a nuclear RNAi(-like) mechanism, we used heterozygote DM1 myoblasts that had approximately 3,200 CTG repeats in one allele of the DMPK gene and 18 CTG repeats on the other (Fig. 1A), as verified by Northern (Fig. 1B) and Southern blots (data not shown). The DM1 myoblasts were isolated from the quadriceps of a 15-week-old aborted congenitally affected human DM1 fetus. The congenital form of DM1 fetus was confirmed by hypermethylation proximal to the CTG expansion (data not shown). A lentivirus-based vector (pHIV7) was used to deliver the shRNAs targeting DMPK mRNAs under the control of the human U6 Pol III promoter into the DM1 myoblasts (*13*). An eGFP gene driven from a CMV promoter was also incorporated as a selection marker (Fig. 1C). Four shRNAs were designed: two are complementary to the coding sequences of the DMPK mRNA (DM10 and 130), one is targeted to a proven accessible target site in 3'UTR (DM1892) (*14*) and one targets an irrelevant sequence (CTRL IR) (Fig. 1D). ShRNA constructs were efficiently transduced into cultured DM1 myoblasts (Fig.2A). High levels of transgene expression could be obtained throughout myoblast differentiation following their genome integration (*15*). The cells expressing eGFP were then sorted by flow cytometry. The expression of shRNAs was assessed using the total RNAs isolated from sorted cells by Northern hybridization with sense probes that hybridize to the antisense strands, but not to targets (Fig. 2A). All shRNAs were expressed from the lentiviral vectors and they were also processed into approximately 21-nt siRNAs (Fig. 2 A).

In order to investigate function of shRNA constructs in DM1 myoblasts, total RNA was extracted from all five lentiviral transduced primary myoblast cultures, and the level of reduction of DMPK mRNA expression was determined by Northern hybridization using a radiolabeled DMPK cDNA probe (Fig. 2B). Since normal DMPK mRNA contains between 5 and 37 CUG repeats, it is easily distinguished by Northern analyses from the mt DMPK

transcripts containing long CUG repeats (Fig. 1B). Myoblasts were allowed to differentiate in the absence of growth factors for 48h prior to RNA extraction in order to induce the expression of DMPK mRNA (*16*). Steady-state levels of mt transcripts were elevated in congenital DM1 cells because mt DMPK transcripts with large CUG repeat expansions were more stable than the normal transcripts (data not shown). ShRNAs DM10 and DM130 showed the most effective down-regulation of normal (wt) DMPK mRNAs (64.2 ± 3.5% and 74.5 ± 2.3% decrease in expression relative to control expression, respectively) (Fig. 2C). Unexpectedly, they also effectively down regulated mt DMPK transcripts that are localized exclusively in the nucleus (46.6 ± 2.4% and 57.6 ± 6.4%) (Fig. 2, B and C). The shRNA directed against the 3'UTR (DM 1892) showed only a moderate decrease in expression of both wt and mt DMPK transcripts (26.5 ± 2.4% and 15.1 ± 3.3%). This result is in accordance with several reports showing that RNAi is not as effective against the target sequences located in the 3'UTR of transcripts (*17*). The irrelevant shRNA control did not show any significant alterations in total DMPK mRNA levels. Taken together our data suggests that RNAi (-like) phenomenon occurs not only for cytoplasmic transcripts, but also for nuclear transcripts.

To confirm that nuclear transcripts are affected by shRNAs, we performed nuclear and cytoplasmic fractionation of differentiated DM1 cells stably expressing the shRNAs (Fig. 3, A to C). The mt DMPK mRNAs were observed exclusively in the nucleus (Fig. 3A). The nuclear-retained mt mRNAs were reduced effectively by both shRNAs DM10 and DM130 (51.5 ± 6.6% and 48.8 ± 3.9%) (Fig. 3C). In addition the normal transcripts remaining in the nuclear fraction were also reduced by the shRNAs (47.2 ± 5.7% and 46.2 ± 4.0%). As expected, shRNAs DM10 and DM130 down regulated the normal transcripts found in the cytoplasm (77.8 ± 1.3% and 73.6 ± 1.5%). We could not detect any intermediary cleavage products in nuclear targeted transcripts, indicating that the RNAs are rapidly degraded following cleavage. The changes in DMPK levels obtained from this fractionation experiment showed a similar trend as that seen with the Northern blot in Figures 2B and 2C. ShRNAs DM10 and DM130 are the most effective and DM1892 has a weak but significant effect. This result demonstrates that shRNAs can reduce nuclear transcripts.

Some defects seen in DM1 patients are thought to originate from haplo-insufficiency of the DMPK protein, meaning that only the wt transcripts can be translated into proteins since all mt DMPK mRNAs are retained in the nucleus (*18*). To confirm this, a Western blot was performed to measure the levels of DMPK protein translated from normal transcripts. ShRNAs DM10 and 130 reduced DMPK protein levels by 55.9 ± 3.2% and 73.2 ± 3.0% respectively relative to the control (Fig. 3, D to F). The levels of DMPK protein are lowed by 30.8 ± 4.7% in DM1 cells expressing shRNA DM1892. These results are consistent with the reduction in mRNA levels determined by Northern blotting (Fig. 2, B and C). A reduction in steady-state mRNA levels may not depend on RNA degradation but may rather be caused by a decrease in transcription since PTGS and TGS can be initiated by dsRNAs in directing the same degradation pathway (8).

To further investigate this possibility we carried out nuclear run-on assays on nuclei isolated from our transduced DM1 myoblasts expressing the shRNAs. The transcriptional levels of both mt and wt DMPK transcripts were not altered in the myoblasts expressing the different shRNAs (Fig. 4), ruling out changes in transcription. The reduced mRNA levels thus result from PTGS in both the nucleus and the cytoplasm of DM1 cells.

A nuclear RNAi pathway was predicted by a study that the shRNAs under control of the U6 promoter are almost exclusively nuclear (19). Cullen and his colleagues also documented that siRNAs partially reduced the nuclear level of the target mRNAs even though they claimed that RNAi in human cells is restricted to the cytoplasm (5). They were suspicious about their nuclear mRNAs that may be in the process of being exported and may not be truly nuclear.

The mt DMPK mRNAs, however, are exclusively located in the nucleoplasm [this study and (11)], and they are clearly down regulated by appropriate shRNAs. The demonstration that substrates for shRNAs are destroyed in the nucleus raises the possibility that shRNAs may be processed into siRNAs by a separate enzymatic activity from that found in the cytoplasm. Further detailed investigations will be required to distinguish the differences between nuclear and cytoplasmic RNAi components, however, a new nuclear RNAse III activity, Drosha with similarities to Dicer has been identified (20) and may be important for the effects we see. Nuclear localized human Drosha is an essential component for miRNA processing (*20*). The sequences homologous to human Drosha have also been found in *C. elegans*, *D. melanogaster*, and mouse, indicating its widespread conservation (21). Data from studies in *C. elegans* also showed a partial decrease in the level of nuclear mRNAs during RNAi (22).

Recent reports have also demonstrated that some vectors containing shRNAs induce an interferon response, rather than RNAi activity (*23, 24*). This is an unlikely occurrence in our system because our controls did not show reduction of DMPK transcripts, nor non-specific alteration of transcription or translation as would be expected with an interferon response or PKR activation (*24, 25*). Several double-strand RNA binding proteins (DRBP) with PTGS activity other than Dicer have been identified in the nucleus of mammalian cells (25). However, most of these nuclear proteins are believed to function in the dsRNA-induced transcriptional repression rather than target-specific RNA degradation (25).

The demonstration of specific target mRNA cleavage by shRNAs in the nucleus of human cells is of particular importance for several reasons. Aside from challenging the current dogma that message degrading RNAi mechanisms function solely in the cytoplasm, it suggests the possibility of targeting pre mRNAs. The co-existence of an innate immune system and RNAi in mammalian cells may be required for both maintenance of genome integrity as well as antiviral defence. The existence of a molecular mechanism for innate immunity mediated by DNA deamination has recently been demonstrated (*26, 27*). Our

results suggest that the RNAi mechanism may also play a role in host response to double-strand RNAs in the nucleus.

5.4. Materials and methods

Primary human muscle cell cultures

DM1 and normal control myoblasts were obtained from the quadriceps of 15 week-old aborted fetuses. The congenital DM1 fetus had approximately 3,200 CTG repeats on the mutant allele, as verified by Southern and Northern blotting. Skeletal muscle biopsies were approved by Laval University and the CHUL's ethical committees. Myoblasts were grown in MCDB-120 (Clonetics®) supplemented with 15% heat-inactivated fetal bovine serum, 5 μg/ml insulin, 0.5 mg/ml BSA, 10 ng/ml human hrEGF, 0.39 μg/ml dexamethasone, 50 μg/ml streptomycin and 50 μg/ml penicillin. Differentiation was carried out in DMEM supplemented with 0.5% heat-inactivated fetal bovine serum, 10 μg/ml insulin, 10 μg/ml apo-transferrin, 50 μg/ml streptomycin and 50 μg/ml penicillin.

Cloning of shRNAs into pHIV7 and transductions of DM1 cells

ShRNAs DM10, 130 and 1892 are directed to nucleotides 10 to 30, 130 to 150 and 1892 to 1912 of the human skeletal muscle DMPK mRNA (Genebank accession number: NM_004409), respectively. ShRNAs were expressed under the transcriptional control of a human RNA Pol III (U6) promoter element using a fast and simple cloning method (unpublished data). The 5' PCR primer contains the sequences of the 5'-end of the U6 promoter, and the 3' primer contains the complementary sequences of the 3'-end of the U6 promoter and shRNA, and six adenines. The PCR products have a 3' A overhang that can be ligated into the pCR2.1 TA cloning vector (Invitrogen). The shRNA clones were confirmed by DNA sequencing.

The shRNA constructs in pCR2.1 vectors were digested with BamH1-EcoRV and subcloned into the BamH1-Sma1 site of the pHIV7 vector (*13*). 293T cells were co-transfected with the pHIV7, pCMV-Rev, pGP (coding for gag and pol) and pCMV-G (coding VSV-G). Infectious lentivirus particles pseudotyped with VSV-G were collected

after 48h and incubated for 30 minutes with DM1 myoblasts of low passage (P<6). Transduced cells expressing eGFP encoded in pHIV7 were isolated 3 days later by FACsort. ShRNA and target gene expression were revealed by Northern hybridization in eGFP-positive DM1 myoblasts harbouring integrated lentiviral vectors.

DMPK mRNA extraction

Proteinase K digestion of DM1 cells is the most appropriate method to completely extract mt DMPK mRNAs that are bound to the nuclear matrix (unpublished data). Three ml of fresh lysis buffer were added to confluent myoblasts differentiated for 2 days in a 60mm dish. The lysis buffer contained the following: 500 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1.5 mM $MgCl_2$, 10 mM EDTA, 2% SDS and 0.5 mg/ ml of proteinase K. The cells were harvested after detaching from the dishes and incubated at 45^{o}C for 45 minutes in a shaking water bath. Total RNAs and DNAs were then recovered in 100µl water by standard acid phenol-chloroform extraction. One ml of Trizol (Life Technologies) was then added to the samples. RNA was extracted according to the manufacturer's specifications.

Northern blotting

Ten micrograms of total RNA were loaded in a 1.2% agarose gel with 2.2M formaldehyde and 1 X MOPS. Samples were separated by electrophoresis and then transferred to a Biodyne B nylon membrane (Pall Corp., Ann Arbor, MI) using a microfiltration apparatus (Pharmacia). Hybridization conditions were previously described (*14*). The labelled DMPK probe was prepared by random priming of 50 ng of the BglII-SacI fragment of the DMPK cDNA. The cyclophilin probe was prepared by random priming of a 700 bp PCR product amplified from cyclophilin cDNA. Densitometry analyses were performed on the original autoradiograms with an AlphaImager imaging system and software (Innovatech Corp.). Sample normalization was based on cyclophilin expression levels within each cell type using the level of normal transcripts in the CTRL cells as the baseline.

For detection of shRNAs, Northern hybridization was performed as previously described (28). Total RNAs were obtained by addition of Trizol to the harvest cells according to the manufacturer's instruction. Twenty micrograms of total RNA were loaded in a 6 % denaturing polyacrylamide gel. RNAs were transferred by electroblotting on a nylon membrane. To detect shRNAs, the radiolabeled 21-mer sense strand oligos for each shRNA were used. U6 snRNA was also probed and served as an RNA loading standard.

Western blotting

DM1 cells were grown to confluence in a 100 mm dish and scraped into differentiation media after 48 hours of growth. The cell pellet was washed twice in HBSS and the protein content was quantified by BCA protein assay (Pierce). Forty micrograms of protein were resolved in a SDS-PAGE and electroblotted to a nylon membrane. Blotting conditions have been described previously (*18*). The anti-DMPK polyclonal antibody was a gift from Lubov Timchenko (Baylor College, Tx). Normalization for DMPK protein level was carried out by quantifying total proteins transferred to the nylon membrane as a loading standard.

Cell fractionations

Differentiated myoblasts were collected and nuclear and cytoplasmic fractionations were carried out using digitonin lysis buffer as described previously (*29*). Total RNAs from the nuclear pellets and the cytoplasmic fractions were extracted with 2 ml of proteinase K buffer as described earlier. Five micrograms of total RNA from each fraction were loaded in a 1.2 % agarose gel with 2.2 M formaldehyde and 1 X MOPS. It was necessary to load approximately four times the volume of nuclear RNA to equal the amount of one volume of the cytoplasmic RNA. Normalization was performed using total RNA in the gel prior to transfer as a loading standard.

Slot Blots and Nuclear Run-on assays

Three μl of 5 M NaOH and water were added to each probe to be 50 μl: 125 ng of CTG-80 probe (240 bp), 727 ng of the DMPK probe (1454 bp), 5 μg of pBluescript KS+ vector, or 350 ng of cyclophilin probe (700 bp). The probes were then boiled for 5 minutes and cooled on ice. After addition of fifty μl of 4M ammonium acetate, each probe was applied to a nylon filter inserted in a slot-blot apparatus. After gentle suction, slots were washed twice with 2 X SSC. The nylon filter was removed and UV-crosslinked for 3 minutes for nuclear run-on assays.

For nuclear run-on assays, isolation of nuclei and synthesis of radiolabeled nascent RNAs were performed as described previously (*30*) with the following modifications. Transcriptions were carried out for 10 minutes in the presence of 200 μCi ^{32}P-ATP per sample to maintain similar specific activities between wt and mt DMPK transcripts. Total labelled RNAs were extracted from the samples described above. Hybridization was carried out overnight in the same Northern blot hybridization buffer as described previously (*14*), except replacing salmon sperm DNA with 200 μg/ml of *E. coli* tRNA. The CTG-80 probe specifically binds to mt DMPK mRNA but not to wt transcripts, as verified by Northern blotting (Data not shown). All assays were performed in duplicate and carried out simultaneously with or without 400 μg/ml α-amanitin, which blocked transcription in all samples (data not shown). DMPK mRNAs were normalized with cyclophilin transcripts in each sample.

5.5. Acknowledgements

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5.7. Figure legends

Figure 1. Human DMPK mRNAs in DM cells and anti-DMPK shRNAs in lentiviral vectors. (A) Illustration of the human DMPK mRNA and relative positions of different shRNAs. DM1 myoblasts have a normal (wt) allele with approximately 18 CTG repeats and a mutant (mt) allele with approximately 3,200 CTG repeats in the 3'UTR of the DMPK gene. The shRNAs against DMPK mRNAs are designated as the first nt of their target sequences from the initiation codon. **(B)** Northern blot showing DMPK mRNA expression in normal and DM1 differentiated myoblasts. In normal myoblasts, DMPK alleles had 14 and 17 CTG repeats in the 3'UTR of the DMPK gene, respectively. These mRNAs cannot be separated, and appear as a single 2.8 kb band. DM1 myoblasts have a wt allele with 18 CTG repeats and a mt allele with approximately 3,200 CTG repeats. Theses appear as separate 2.8 kb (wt) and 12.4 kb (mt) bands on the autoradiogram. **(C)** Illustration of HIV-based self-inactivating lentiviral vector used in this study (*13*). The viral core sequence contains a Rev-response element (RRE) followed by a Flap element. The shRNA with 4 loops (TGCG) is expressed under the human U6 promoter and terminated by 6 thymidines downstream of the shRNA. The human cytomegalovirus promoter (CMV) drives eGFP transcription followed by the woodchuck hepatitis virus response element (WPRE). The 3' long terminal repeat (3' LTR) has a complete deletion of the HIV promoter and enhancer sequences (400 bp). **(D)** Sequences of shRNAs. ShRNAs DM10, 130 and 1892 consist of 21-nt sense and antisense sequences directed to nts 10 to 30, 130 to 150 and 1892 to 1912 of the human skeletal muscle DMPK mRNA (Genebank accession number: NM_004409), respectively, with 4 loops in the middle. The control vectors either do not contain any shRNA (CTRL), or contain the irrelevant shRNA (CTRL IR) directed to nts 143 to 163 of a rare isoform of DMPK mRNA (Genebank: L00727) not detectable by RT-PCR in our cells (Data not shown).

Figure 2. Expression of shRNAs and their targets in human DM1 cells transduced with lentiviral vectors. (A) Expression of shRNAs in DM1 cells. Transduced and sorted

DM1 cells were grown to confluence in a 100mm dish and harvested. Total RNA samples were extracted by addition of Trizol to the harvest cells. In (A), combined Northern hybridization results using sense probes for DM10, DM130, CTRL IR, and DM1892 are presented. Total RNA samples from non-transduced cells (Mock) or cells transduced with HIV7 vector alone (CTRL pHIV) cells were used as controls. Human U6 RNA was probed as an internal control for each experiment. A radiolabeled 21-nt oligo was used as a marker (M). **(B)** Expression of both normal and mutant DMPK mRNAs are reduced by shRNAs. Total RNAs were extracted from the different lentiviral transduced DM1 cells and a Northern blot was performed. Myoblasts were allowed to differentiate for 48h in the appropriate media prior to RNA extraction in order to induce DMPK mRNA levels. The membrane was hybridized simultaneously with ^{32}P-labeled DMPK and cyclophilin cDNA probes. **(C)** Quantification of DMPKmRNAs normalized to cyclophilin mRNA shows that wt and mt DMPK levels are reduced in myoblasts expressing shRNAs DM1892, DM10 and DM130. ShRNAs DM10 and DM130 show the best cleavage efficiency. Standard errors are based upon four independent experiments.

Figure 3. Cell fractionation assays and reduction of DMPK protein levels. (A) Differentiated myoblasts were harvested and cytoplasmic (C) and nuclear (N) extracts were prepared using digitonin detergent. Equal amounts of total RNAs were loaded in the gel. This necessitated about four times the volume of nuclear extract compared to cytoplasmic extracts to achieve equal loading of total RNAs. This experiment shows that mt DMPK mRNA is exclusively nuclear. **(B)** GAPDH hybridization demonstrates fractionation purity. **(C)** Quantification of DMPK mRNA within each fraction based on **(A)**. Normalizations were carried out according to total 28s and 18s rRNA intensities in the ethidium bromide-stained gel (data not shown). Mutant (mt) DMPK mRNAs located exclusively in the nuclei are reduced in the cells transduced with shRNAs DM10 and DM130, and to a lesser extent with DM1892. Normal (wt) DMPK mRNAs are also reduced mainly in cytoplasmic (C) fractions and partly in nuclear (N) fractions of cells transduced with shRNAs DM10 and DM130, and to a lesser extent with DM1892. Standard errors are based on three independent experiments. **(D)** Western blotting was performed on whole cell extracts. Cells

were allowed to differentiate 48h prior to harvesting. Only normal DMPK mRNA contributes to DMPK protein levels since the mutant mRNA is completely retained in the nucleus. **(E)** Ponceau red staining of the membrane prior to blotting with a rabbit anti-DMPK antibody. Samples were normalized to total sample protein levels. **(F)** Results show that protein levels are reduced by 55.9 ± 3.2% and 73.2 ± 3.0%, respectively, when DM1 cells express shRNAs DM10 and 130. Standard errors are based on four independent experiments.

Figure 4. Nuclear run-on assays. (A) Nuclei from DM1 cells were extracted and added to transcription buffer containing 200 μCi of ^{32}P-ATP. Transcription was carried out for 10 minutes at 30^{o}C. Total RNAs were extracted from samples and hybridized to slot blots. Slot blots were performed in duplicate, from top to bottom: a probe containing 80 CTG repeats, a DMPK coding sequence cDNA probe, the pBluescript KS+ vector and cyclophilin cDNA. Transcription reactions in the presence of 500ug/ml of α-amanitin were performed in parallel as negative controls. No signals were present in these slot blots (data not shown). **(B)** Quantitation shows that transcription levels of normal (wt) and mutant (mt) DMPK transcripts were not altered by shRNA expression. Standard errors are based on three independent experiments.

5.8. Figures

FIGURE 1

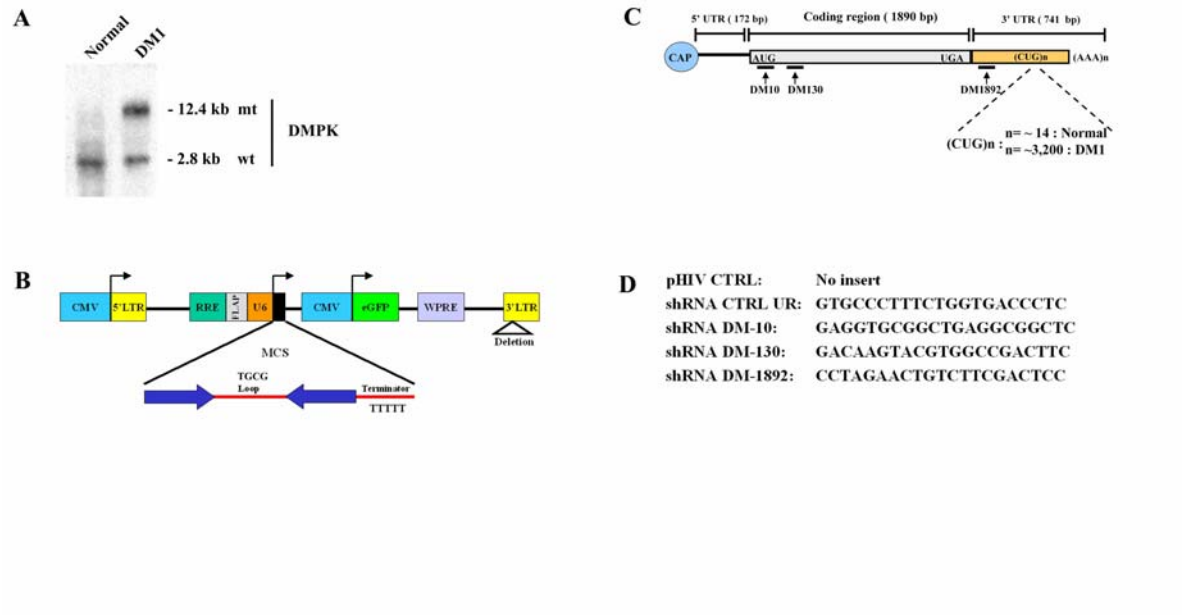
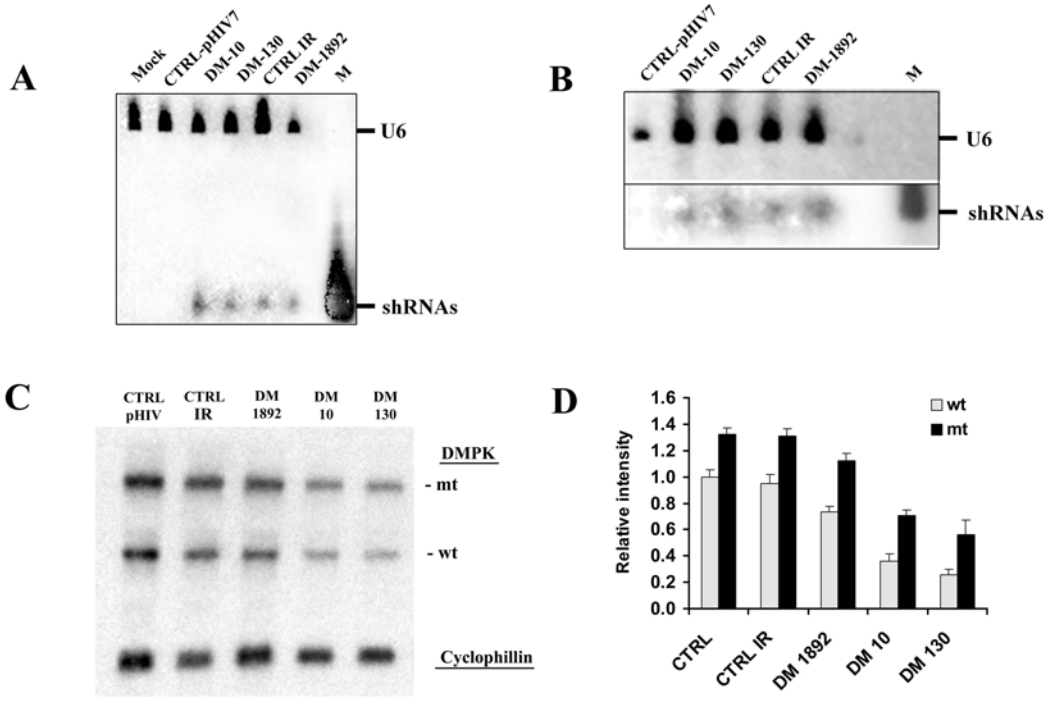


FIGURE 2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CTRL IR
DM-1892
M
U6
shRNAs
C
CTRL pHIV
CTRL IR
DM 1892
DM 10
DM 130
DMPK
- mt
- wt
Cyclophillin
D
Relative intensity
wt
mt
1.6
1.4
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FIGURE 3

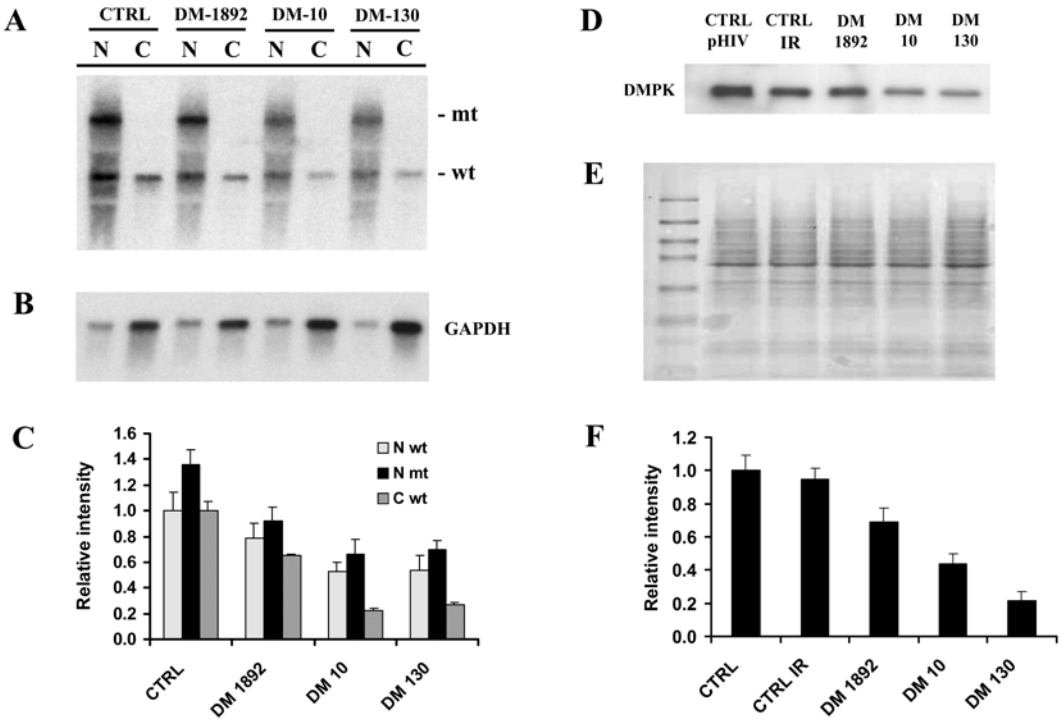
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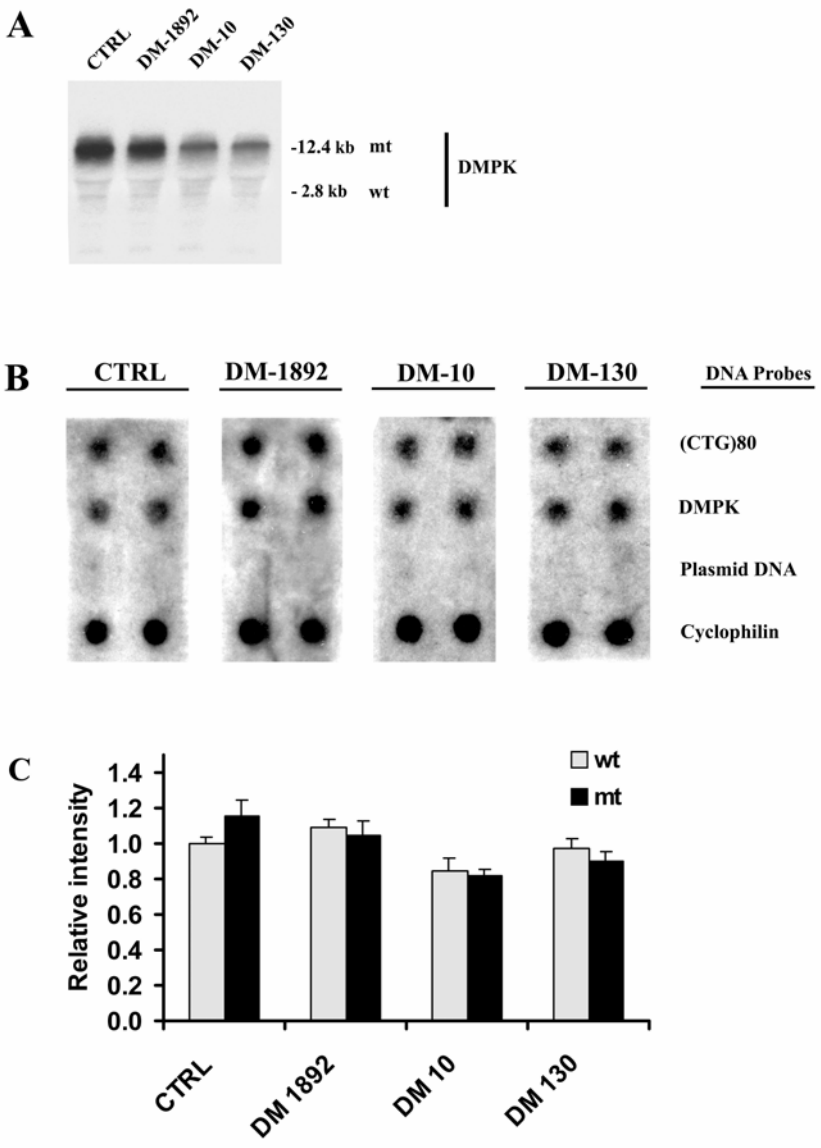
FIGURE 4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C
wt
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Relative intensity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CTRL
DM 1892
DM 10
DM 130

CHAPTER VI

An alternative splicing defect in Myotonic Dystrophy type 1 (DM1) myoblasts can be rescued by overexpression of hnRNP H.

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Manuscript in preparation.

6.1. Résumé

La dystrophie myotonique de type I (DM1) est une maladie neuromusculaire autosomale dominante. Elle est causée par un expansion de CTG dans la région 3'non-codante du gène de la dystrophica myotonica protéine kinase (DMPK). Le mécanisme moléculaire responsable de cette maladie est lié à une perturbation dans l'ARN. Les expansions de CUG forment des structures à doubles-brin capables de recruter des facteurs nucléaires impliqués dans l'épissage des ARNm. En utilisant une méthode permettant de fixer protéines et ARNs par l'intermédiaire d'une irradiation aux rayons ultra-violets, nous avons pu identifier une protéine liant ces ARNs double-brin. HnRNP H est un facteur impliqué dans l'épissage de plusieurs gènes qui se lie de façon spécifique aux ARNs ayant de grandes expansions de CUG. De plus, nous démontrons que cette protéine peut se multimériser lorsqu'elle est en présence de grandes expansions et de séquences régulatrices d'épissage proximales. La surexpression de hnRNP H dans des myoblastes DM1 permet de restaurer un défaut d'épissage du récepteur à l'insuline ainsi que de retrouver des niveaux normaux de CUG-BP. La déplétion de hnRNP H par liaison aux expansions de CUG pourrait donc engendrer les défauts d'épissage caractéristiques du muscle squelettique DM1. Le rétablissement d'un niveau basal de hnRNP H chez les patients souffrant de DM1 pourrait être à la base d'une thérapie génique potentielle.

6.2. Abstract

Myotonic dystrophy type 1 (DM1) is an autosomal dominant neuromuscular disorder associated with a (CUG)n expansion in the 3' UTR region of the DMPK (DM1 protein kinase) gene. The RNA dominance model has been proposed as a molecular mechanism of the disease. The expanded CUG repeats form RNA hairpins that sequester nuclear factors whose depletion are the likely cause of alternative splicing defects seen in several mRNAs. In this report, a modified UV cross-linking assay was used to identify hnRNP H as a major binding protein to the repeats that contributes to the retention of mutant DMPK mRNA in the nucleus. The binding of hnRNP H is specific to (CUG)n RNAs and is proportional to the size of the triplet repeats expansion. Moreover, multimerization of the protein was observed when a splicing branch point is present downstream from the repeat expansion. Overexpression of hnRNP H in DM1 myoblasts provided further evidence that this protein may be depleted in the disease since defective insulin receptor mRNA alternative splicing was rescued in congenital DM1 myoblasts and CUGBP levels were restored to normal. Here we report an important finding for the understanding of the molecular mechanisms that lead to DM1 and set forth the possibility of a novel therapy for the disease.

Key words : Myotonic dystrophy, hnRNP-H, DMPK, CUG repeats.

6.3. Introduction

Myotonic dystrophy type 1 (DM1) is an autosomal, dominantly inherited neuromuscular disorder with a global incidence of 1 per 15000 (Harper, 2001). Adult onset DM1 is primarily characterized by myotonia, muscle wasting, and weakness, but also affects a number of organs and tissues resulting in cataracts, cardiac conduction abnormalities, testicular atrophy, male baldness, and insulin resistance (Harper, 2001). On feature of the disease is the presence of severe congenital (CDM) cases present at birth. The main characteristics of CDM are hypotonia, mental retardation, delayed muscle maturation, and developmental abnormalities. The mutation responsible for the disease is a (CTG)n repeat expansion in the 3' un-translated region of DM protein kinase (DMPK) gene (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). This repeat ranges in size from 5 –37 repeats in the normal population, generally between 50-1000 repeats in adult onset cases, and over 1000 repeats in CDM patients (Harper, 2001).

Several features of DM1 pathogenesis can be explained by an aberrant alternative splicing defect in five different pre-mRNAs which are: cardiac troponin T (cTNT), insulin receptor (IR), muscle-specific chloride channel (CIC-1), tau, and myotubularin-related 1 (Faustino and Cooper, 2003). Misregulation of IR and CIC-1 splicing causes two common symptoms of DM1, insulin resistance and myotonia, respectively. Patients with DM1 have an unusual form of IR that causes defects in skeletal muscle (Furling, 1999). It was shown that alternative splicing of the IR pre-mRNA is aberrantly regulated, resulting in predominant expression of the lower-signaling non-muscle isoform (IR-A)(Savkur et al., 2001a). It was also shown that loss of CIC-1 mRNA and protein in DM1 is due to aberrant splicing and results in skeletal muscle membrane hyperexcitability and myotonia (Charlet et al., 2002).

Among several proposed molecular mechanisms, the RNA dominant mutational model proposes that triplet repeat expansion causes a gain-of-function at the RNA level, possibly

by sequestering an essential cellular protein factor (Caskey et al., 1996; Fardaei et al., 2002; Miller et al., 2000; Timchenko and Caskey, 1996). Targeting and destruction of mutant DMPK mRNA releases these factors thus allowing restoration of several of the disease features (Furling et al., 2003; Langlois et al., 2003). Transgenic mice containing CUG repeats in an unrelated mRNA display myotonia and myopathy phenotype, which supports this idea (Mankodi et al., 2000). It was also demonstrated in a cell culture model system that the (CUG)n repeats as well as the sequence region proximal to the repeats are important for inhibition of myoblast differentiation (Amack and Mahadevan, 2001; Amack et al., 2002). Several CUG repeat binding proteins have been identified including muscleblind, CUG-BP, ETR-3, and PKR (Lu et al., 1999; Miller et al., 2000; Tian et al., 2000; Timchenko et al., 1996). The muscleblind protein binds to CUG repeats in a proportional manner, and showed that it is co-localized to the foci, together with its alternative splicing forms (Fardaei et al., 2002; Miller et al., 2000). But it is still not known how these proteins are involved in alternative splicing defects and DM1 pathogenesis (Savkur et al., 2001a; Timchenko et al., 2001). Although CUGBP does not co-localize with the foci, it has been shown that its expression level is increased in DM1. Functional analyses indicate that increased expression of CUGBP is responsible for the aberrant regulation of cTNT, IR, and CIC-1 splicing (Faustino and Cooper, 2003). For all three of these pre-mRNAs, CUGBP has been shown to bind to U/G rich motifs in introns adjacent to the regulated splice site (Charlet et al., 2002; Philips et al., 1998; Savkur et al., 2001a; Takahashi et al., 2000). It is not known at this time how the repeat expansion in the mutant DMPK mRNA causes an elevation of nuclear CUGBP.

Using a modified RNA/protein cross-linking assay, we searched for a protein that can bind to the (CUG)n repeats and its distal region. HnRNP H was identified as a major binding protein of the CUG repeat expansion and the branch point sequence of exon 16 located in the distal region. This protein can bind to the target RNA with increased binding affinity proportional to the length of the repeat expansion and can be multimerized in the presence of an unidentified docking molecule. Most significantly, the aberrant splicing defect of the insulin receptor can be restored, even in congenital DM1 myoblasts, by overexpressing the

hnRNP H protein. Overexpression of hnRNP H also restores the elevated expression of CUGBP to that of wild type. This report shows that hnRNP H is one of the key molecules implicated in the molecular mechanisms of the disease and can set the groundwork for a DM1 gene therapy.

6.4. Materials and methods

DNA clones

The DMPK clone containing CUG 100 repeats (pRMK-100) was digested with SacI and EcoRI and cloned into pGEM vector and further subcloned by deletion of *SacI/SacII* fragment. Among several subclones containing variety of CUG repeats, the CUG 46 and 85 RNA were selected after sequencing confirmation. The CUG 129 clone was created using two primers containing either 20 repeats of CTG repeats or 20 of CAG repeats as described [42]. The RNA #2 in Figure 4D was created by PCR reaction using two primers (5' GAACGGGGCTCGAAGCTTCCTT 3' and 5' CTAGACTGGAATTCGGCTTATGGTCAGTGATC 3'), and cloned into pBluescript II sk vector. The #3 RNA was created the same way using another set of primers (5' GAATGTCCTGGTACCCGGGAATAA 3' and 5' CTAGACTGGAATTCGGCTTATGGT 3'), and cloned into the same vector. To create hnRNP H-EGFP fusion gene, hnRNP H was PCR cloned by two primers (5' CAGCCATATGCTCGAGTGATG 3' and 5' CTTTGTTAGCAGCCGGATCC 3'). The PCR product was further cloned into *XhoI/BamHI* site of pLEGFP-C1 vector (Clonetech).

RNA

The RNA was in vitro transcribed using T7 RNA polymerase from the linerized DNA plasmids in 20 ul reaction. For the biotinylated RNA, the 10 % of UTP in the transcription reaction was replaced by the biotinylated UTP (Roche). Each RNA from the transcription reaction was mixed with 80 ul of water and further purified using the MicroSpin^{TM}-G50 column (Amersham Biosciences, Piscataway, NJ).

Extract preparation and hnRNH H purification

The source of HeLa cells and the protocol for the nuclear extract preparation is described elsewhere (Kim, 2003). The total cell extract of HeLa and DM1 cells were prepared as following. Cells ($1X10^8$) were harvested and washed with buffer D and mixed the 100 ul of buffer D and sonicated for 15 sec at 4oC. After 5 min microfuge at 4oC, the supernatants were taken and used as a total extract. The HeLa nuclear extract was used for the purification of the CUG binding protein. The extract was precipitated with 30 % of Ammonium sulfate. The supernatant was harvested after 10 min microfuge at 4oC. Then 20 ug of biotinylated $(CUG)_{46}$ or $(CUG)_{85}$ RNA was mixed and incubated with 300 ul of pre-washed streptavidin conjugated dynabeads for 30 min at room temperature. The beads were washed with 1 ml of PBS for 5 times and incubated with 500 ul of extracts for 1 hour at room temperature with slow rocking. The beads were washed with 5 times of 1 ml of buffer D and sequentially eluted with 50 ul of 0.2 M KCl solution. The purified peptides were sequenced by mass spectrometry in the protein sequencing facility at the City of Hope.

100 ul of anti-hnRNP H antibody was incubated with 200 ul of the Protein Conjugated dynabeads at room temperature for 1 hour. The beads were washed with 1 ml of PBS for 5 times and incubated with 50 ul of the nuclear extract at 40C for 1 hour. The mixture was spun for 5 min using the microcentrifuger, and the supernatant was used as an immuno-depleted extract of hnRNP H. The depletion of hnRNP H was confirmed by Western blot using the anti-hnRNP H antibody. For the reconstituted extract, 20 ul of the hnRNP H depleted extract was mixed with 20 ng of hnRNP H recombinant protein and incubated at room temperature for 10 min.

Immunodepletion of hnRNP H

100 ul of anti-hnRNP H antiserum was incubated with 200 ul of the Protein A conjugated Dynabeads at room temperature for 1 hour. The beads were washed with 1 ml of PBS for 5 times and incubated with 50 ul of the nuclear extract at 4^oC for 1 hour. The mixture was

spun for 5 min, and the supernatant from the supernatant was used as an immuno-depleted extract of hnRNP H. The depletion of hnRNP H was confirmed by the Western blot using the anti-hnRNP H antiserum. For the reconstituted extract, the 20 ul of the hnRNP H depleted extract was mixed with 20 ng of recombinant hnRNP H protein and incubated at room temperature for 10 min.

In vitro and in vivo cross linking assay

A total 10 ul of extract was mixed with E. coli tRNA (Sigma) at a final concentration of 2 mg/ml and incubated at room temperature for 10 min. 1 ul of G50 column purified RNA was mixed and incubated at room temperature for 20 min. The reaction was put on a petri dish floating on ice cold water and UV-irradiated using a Stratalinker 2400 (Stratagene, San Diego, CA) 5 cm from the light source for 10 min.. Samples were digested with 10 ug of RNAse A for 10 min at 37^{o}C and resolved on a 10 to 15 % SDS-PAGE gel. For the *in vivo* UV cross linking the details are described in elsewhere (Kim, 2003).

RNAi assay

For synthesis of three siRNAs against to hnRNP H, two sets of oligos were designed (AS1; 5' AAGGTGGAGAGGGATTCGTGGCCTGTCTC 3', S1; 5' AACCACGAATCCCTCTCCACCCCTGTCTC 3', AS2; 5' AACTTGAATCAGAAGATGAAGCCTGTCTC 3', S2; 5' AACTTCATCTTCTGATTCAAGCCTGTCTC 3'). Each siRNA was synthesized using silencer siRNA construction kit from Ambion. For siRNA assay, 293 or DM1 cells were transfected with 100 ug of EGFP-(CUG)85 reporter gene and either one of anti-hnRNP H siRNA or irrelevant siRNA (EGFP #2) in final 10 nM using lipofectamine 2000.

Native gel

Non-denaturing composite gel electrophoresis was performed as described (Kim et al., 1999). The reaction was mixed with an equal volume of native loading dye containing bromophenol blue and 50% of glycerol.

Primary human skeletal muscle cell cultures

DM1, CDM1 and normal control myoblasts were obtained from the quadriceps of 15 week-old aborted fetuses. The DM1 fetus had approximately 750 CTG repeats and the CDM1 fetus had approximately 3000 CTG repeats, as verified by southern blot. Skeletal muscle biopsies were approved by Laval University and the CHUL's ethical committees. Myoblasts were grown in MCDB-120 supplemented with 15% heat-inactivated fetal bovine serum, 5 μg/ml insulin, 0,5 mg/ml BSA, 10 ng/ml human hrEGF, 0,39 μg/ml dexamethasone, 50 μg/ml streptomycin and 50 μg/ml penicillin. Differentiation was carried out in DMEM supplemented with 0,5% heat-inactivated fetal bovine serum, 10 μg/ml insulin, 10 μg/ml apo-transferrin, 50 μg/ml streptomycin and 50 μg/ml penicillin.

Myoblast transfection

Normal, DM1 and CDM1 myoblasts were grown in 100 mm plates (Sarstedt) until they reached 70% confluence. They were then trypsinised and seeded at 2.5 $X10^5$ cells per well in a 6 well plate. The next day, the cells were harvested, washed twice in HBSS and transfected with 2 μg of purified CMV-hnRNP-H-eGFP or the control CMV-eGFP plasmids using the human dermal fibroblast nucleofector kit (Amaxa) and program P-22. Transfection resulted in over 70% eGFP positive cells that were sorted 24h later by flow cytometry. Sorted myoblasts were plated in proliferation media for 24h before being placed in differentiating medium.

Detection of insulin receptor splicing

Sorted myoblasts were allowed to differentiate for 4 days and total RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer's specifications. First strand cDNA synthesis and PCR amplifications of the insulin receptor A and B isoform cDNAs were done using identical primer sequences and assay conditions as previously reported (Sarkar, 2001). PCR amplification resulted in a 131 bp amplicon for isoform A and a 167 bp amplicon for isoform B. PCR products were resolved on a 2.5% agarose gel and densitometry analysis was done using an AlphaImager imaging system and software (Innotech Corp.).

Myoblast fusion assay

Sorted myoblasts were seeded in a 96 well plate and were allowed to differentiate for 4 days. Cells were then fixed with 4% paraformaldehyde/HBSS for 5 minutes and rinsed with HBSS. Cells were then treated with HBSS containing 1ug/ml DAPI for 5 minutes, rinsed once with HBSS and analysed under a fluorescence microscope.

6.5. Results

UV cross-linking of mutant DMPK 3' UTR binding protein

As a substrate for *in vitro* cross-linking, the *SacII/Pst1* fragment of wild type with (CUG)5 and mutant DMPK genes with (CUG)85 or (CUG)46 were cloned and transcribed in vitro (Fig.1A). The labeled RNA was used for the modified UV cross linking assay using a HeLa nuclear extracts. When the wild type DMPK RNA was used for the assay, we didn't detect any specific cross linking products (Fig 1B. Lane 1). It has been reported by others that there are several binding proteins in this region (Tiscornia and Mahadevan, 2000). Since we used 10-fold more nonspecific RNA to select a protein with strong binding affinity, these proteins may not have bound in our conditions. With the $(CUG)_{46}$ RNA, there was a 50 Kda binding product (lane 3). Interestingly, the binding affinity was increased using RNA with 85 CUGs'. Additional products were observed with approximated molecular weights of 100 and 200 Kda (lane 2). Since molecular weights of the additional products are correspondent to multiples of 50Kda, it is possible that the protein is multimerized with $(CUG)_{85}$ but not with $(CUG)_{46}$. As a possible purification tool, we verified if the protein could bind to RNAs containing biotinlylated UTP. The binding patterns of two different substrate RNAs are identical, indicating that the biotinylation of RNA doesn't inhibit the binding of these proteins (lanes 4 and 5).

Before further identification of the protein, we wondered if the cross linking product can be seen in the extracts made from DM1 myoblasts (Fig. 1C). Total extracts were prepared from HeLa cells and from differentiated and undifferentiated DM1 myoblasts. To make sure that each extract contained similar protein amounts, total amount of proteins in each extracts were adjusted after Commassie blue or silver staining (data not shown). When we used HeLa nuclear extracts, the cross linking products were almost identical to the previous experiments using total cell extracts. When the assay was done using the myoblast extract, the 50 Kda and 100 Kda bands were present using the $(CUG)_{85}$ RNA substrate, but with a

reduced efficiency (lane 4). The two cross-linking products were not present if the shorter $(CUG)_{46}$ RNA was used (lanes 3 and 5). If a differentiated cell extract was used, the cross linking of 50 Kda was further reduced (lane 6). Since the 50 Kda protein is cross-linked both in HeLa and DM1 myoblasts, we decided to pursue further. Since the 35 Kda protein doesn't bind to the longer CUG repeats, it was not investigated.

Purification and identification of the mutant DMPK binding protein

A nuclear HeLa cell extract was prepared, and proteins were precipitated in 30 % of ammonium sulfate. We found that the supernatant of the 30 % salt precipitation had a high binding affinity to the longer $(CUG)_{85}$ repeat. The biotinylated $(CUG)_{85}$ RNA was incubated in the partially purified extract. The RNA/protein complex was purified using streptavidin conjugated beads. The bound proteins were washed and eluted in high salt. Using 200 mM of salt, we can see the elution of the 50 Kda protein using both the shorter and longer CUG repeat RNA substrates (Fig. 2A). The proteins were cut from the SDS-PAGE gel and micro-sequenced using mass spectrometry. The sequencing data showed that the protein is hnRNP H (Fig. 2B).

HnRNP H binds to CUG repeats and is multimerized by association of an additional factor(s)

HnRNP H has been extensively studied as an alternative splicing factor (Caputi and Zahler, 2002; Chen et al., 1999; Jacquenet et al., 2001; Markovtsov et al., 2000). Anti-hnRNP H antibodies were acquired (generous gift from Dr. Black and Dr. Helfman) and used to confirm the identity of the protein (Fig. 3A). We did the cross-linking experiment in the presence and absence of anti-hnRNP H antibody. When the reaction was performed with pre-immune serum, we could see the consistent cross linking results observed previously (lanes 1 and 2). If the reaction was done in the presence of anti-hnRNP H serum, the cross linking products of 50 (1X), 100 (2X), and 200 Kda (4X) disappeared and a 35 Kda product started to cross-link (lanes 3 and 4). The enhanced binding to the longer repeats in

the presence of anti-hnRNP H antibody indicates that the 35 Kda protein is not a major CUG-binding product. Since the molecular weights of the products are 50, 100, and 200 Kda, it is possible that hnRNP H binds to the target RNA and is multimerized as a dimmer or tetramer. Since hnRNP H appears as a single band using $(CUG)_{46}$ in the cross-linking experiment (Fig. 3A), stabilization of the RNA hairpin structure may be essential for efficient binding of hnRNP H.

This study shows that binding of hnRNP H to the CUG repeats is proportional to the length of the CUG repeats. If the protein binds and is multimerized to the target RNA, it will bring strength to the RNA dominant model in which an exacerbation of the phenotype depends on the length of the mutant DMPK mRNAs. Although muscleblind proteins has been shown to bind to the repeat sequence proportionally, none of the CUG- binding proteins previously identified shows any multimerization phenotype (Miller et al., 2000). The cross linking products were immuno-purified using the anti-hnRNP H antibody to further investigate the possibility of multimerization (Fig. 3B). When the anti-hnRNP H antibody conjugated beads were used, both 50 and 100 Kda products can be co-immunopurified. The 35Kda protein is cross-linked but not precipitated by this assay, demonstrating the specificity of the antibody. Our results show that hnRNP H forms at least a dimmer with the substrate RNAs. We then used a native gel to analyze complex formation (Fig. 3C). Heterogeneous complexes indicate binding of hnRNP H to the CUG repeat expansion with multimerization (lane 4). In the presence of anti-hnRNP H antibody, the complex formation was reduced and turned into a supershift product, indicating that the hnRNP H is a component of the complex (lanes 5 to 8). As a negative control a CAG-repeat RNA was transcribed and used in the same assay (lanes 1 and 2). No complex formation was detected.

Recombinant hnRNP H protein was prepared as described (Markovtsov et al., 2000) and assayed for its binding to the CUG repeats. When binding was tested using the UV cross-linking assay, none of the 100 KD or larger products were detected (Fig. 4A). When we

used $(CUG)_5$, $(CUG)_{46}$, and $(CUG)_{85}$ RNAs, different binding patterns were observed in the native gel (Fig. 4B). The recombinant protein did not bind to $(CUG)_5$ (lane 3). Although binding affinity was enhanced with longer CUG repeats (lanes 6 vs. lane 9), the recombinant protein itself did not form the multi-complex as observed in the total extract (lanes 5 vs. 6 and lanes 8 vs. 9). The recombinant hnRNP H shows a higher binding affinity to the longer CUG repeats but failed to form the multi-complex. This may indicate that the recombinant protein has either lost a biochemical character or requires an additional cellular factor to form the multi-complex. The extract depleted of hnRNP H was prepared using anti-hnRNP H conjugated beads and was assayed for complex formation on native gel (Fig. 4C). To make sure of complete hnRNP H depletion, a western blot was done using the extracts before and after the immunodepletion (Fig. 4C). When hnRNP H was depleted in the total extract, there were fewer complexes formed, indicating that hnRNP H is the major RNA binding protein (lanes 2 and 3). When the hnRNP H-depleted extract was mixed and reconstituted with the recombinant hnRNP H protein, the formation of multiple complexes was restored to the corresponding level as seen in unmodified extract (lanes 4 and 5). These data show that the recombinant protein is fully active, and additional cellular factor(s) are required for hnRNP H to be multimerized. This data indicates that hnRNP H-mediated multimerization is a two-step procedure. First, hnRNP H binds to the repeat RNA and forms an RNA/hnRNP H complex. Sequentially the RNA/hnRNP H complex is multimerized by incorporation of an unidentified factor(s) in the complex.

Binding of hnRNP H requires the CUG repeats and a splicing branch point

To identify the minimum *cis* element for hnRNP H binding, several deletion clones were created. The distal region to the CUG repeats could be deleted without effect on hnRNP H binding (Fig. 4D lane 3). The binding of hnRNP H is abolished if the branch point of exon 16 (Tiscornia and Mahadevan, 2000) is mutated or deleted (Fig. 4D, lanes 2). The upstream sequence to the CUG repeats could be deleted without effect on the protein binding (lane 4). Surprisingly enough, the protein did not bind to a longer CUG repeats, $(CUG)_{129}$, without the branch point sequence (lanes 6 and 7). Based on these data, we concluded that

hnRNP H binding requires CUG repeats and the branch point sequence of Exon 16 located downstream to the repeated CUGs'.

Under-expressed hnRNP H binds to mutant DMPK mRNAs in DM1 myoblasts

It has been shown that expression of hnRNP H is regulated and under-expressed in differentiated muscle cells (Liu et al., 2001) . We determined the expression level of hnRNP H in total HeLa and normal or DM1 myoblasts before or after differentiation (Fig. 5A). The protein is expressed about 10 fold less in undifferentiated myoblasts. The expression is further reduced after differentiation. These data were further supported by the *in situ* hybridization assay (Fig. 5B). As shown by others, hnRNP H is exclusively localized in nucleus (Liu et al., 2001). This explains why less hnRNP H cross-linked in the DM1 cell extracts (Fig. 1C). Since a limited amount of hnRNP H is available in myoblasts, especially after differentiation, increased depletion of hnRNP H will occur through binding to the mutant DMPK mRNA *in vivo*.

When eGFP and CUG repeats sequences are fused and expressed in myoblasts, eGFP expression is severely reduced by inhibition of nuclear export of the RNA (Amack and Mahadevan, 2001). Since hnRNP H is a major protein that binds to the expanded CUG repeats, the reduced expression of hnRNP H may restore the reporter gene expression by blocking the nuclear retention of the mutant RNAs by hnRNP H. SiRNA-mediated gene silencing has been well established in multiple organisms including human cells (Elbashir et al., 2001).To suppress the expression of hnRNP H, three siRNAs were designed to target hnRNP H mRNA. Three individual siRNAs were co-transfected with a eGFP-hnRNP H fusion clone to validate the potency of siRNAs in 293 cells. SiRNA #1 and #2 showed a significant restoration of eGFP expression (Fig. 5C). Anti-hnRNP H siRNAs #2 or an irrelevant siRNA were co-transfected into DM1 myoblasts with the eGFP-$(CUG)_{85}$ clone (Fig. 5D). DM1 myoblasts showed a reduced eGFP expression with the irrelevant siRNAs (left panel), but expression was restored by co-transfection of anti-hnRNP H siRNAs (right

panel). This experiment shows that hnRNP H is one of the major proteins that bind to the mutant RNA and is responsible for retaining mutant DMPK mRNAs in the nucleus of DM1 myoblasts.

The interaction between hnRNP H and mutant DMPK RNA was further confirmed using *in vivo* cross-linking (Fig. 5E). Normal myoblasts were transfected with a body labeled $(CUG)_{85}$ and UV-irradiated to cross-link the protein-RNA interaction. HnRNP H was immunopurified from the total extract of the UV irradiated cells using the anti-hnRNP H antibody. The purified beads were vortexed in the presence of phenol and bound RNA was extracted. The RNA was separated on a denaturing gel and a Northern blotting using the labeled $(CAG)_{10}$ probes was performed. This experiment indicates that the *in vivo* interaction between hnRNP H and CUG repeats can be revealed by UV cross-linking and is antibody-specific. Total amounts of immunopurified hnRNP H was compared by Western blot to ensure an equal loading of samples. When the parallel experiment was done using the DM1 cells, we didn't detect the bound RNA (data not shown). HnRNP H in DM1 cells may already be bound to the endogenous CUG repeats and may not be available for binding to the exogenous substrate RNAs.

Over expression of hnRNP H restores an alternative splicing defect in DM1 cells.

Altered alternative splicing has been shown as a characteristic pathological phenotype of DM1 myoblasts (Charlet et al., 2002; Faustino and Cooper, 2003; Savkur et al., 2001a). If hnRNP H is an essential protein depleted in DM1 by binding to the mutant (CUG)n repeats, limited hnRNP H may be the reason for the defect in alternative splicing, supplementing extra hnRNP H might correct the defective phenotype. Insulin resistance observed in DM1 skeletal muscle results in expression of a lower signaling IR isoform (IR A) (Savkur et al., 2001a).

DM1 myoblasts containing 750 CUG repeats were transfected with the hnRNP H-eGFP fusion gene under the CMV promoter, and the transfected cells were sorted. When normal myoblast were transfected with either eGFP or eGFP-hnRNP H fusion gene, no change was observed in IR alternative splicing or in cell morphology, suggesting a nontoxic effect of hnRNP H overexpression (Fig. 6A, lanes 1 and 2, and data not shown). When the alternative splicing pattern of DM1 myoblasts was tested, the typical aberrant splicing pattern was detected (lane 3). The lower signaling isoform (IRA) is more expressed than isoform IRB. Most strikingly, the splicing pattern can be restored to wild type levels if hnRNP is overexpressed (lane 4). The expression of hnRNP H was confirmed by Western blotting (Fig. 6C). The idea using overexpression of hnRNP H as a possible gene therapy reagent in DM1 was further tested using CDM1 myoblasts with 3000 CUG repeats. The identical experiment was done and shows a partial rescue of the defective splicing phenotype (Fig. 6B). Unlike DM1 myoblasts with 750 CUGs', the restoration of alternative splicing in CDM1 myoblasts is not quite complete, showing less IRB than IRA. Even after overexpressing hnRNP H, no significant differences were observed in foci formation (data not shown).

Overexpression of hnRNP H restores the expression level of CUG-BP1

It has been demonstrated that the steady-state level of the splicing regulator CUG-BP is elevated in DM1 patients (Savkur et al., 2001a; Timchenko et al., 2001). For both cTNT and IR pre-mRNAs, CUG-BP binds to specific intronic RNA elements *in vitro* and induces aberrant splicing when overexpressed in normal cells (Philips et al., 1998; Savkur et al., 2001a). To investigate the role of hnRNP H in the overexpression of CUG-BP, we used DM1 myoblasts overexpressing hnRNP H and tested to see if the restoration of the alternative splicing phenotype coincides with restored expression of CUG-BP. Western analysis of whole cell protein extracts using antibodies against CUGBP shows that the total levels of the protein are increased by three fold as shown by others (Fig. 6C) (Philips et al., 1998; Roberts et al., 1997; Timchenko et al., 1996). When the cells overexpress hnRNP H,

the total amount of expressed CUGBP is restored to the level of normal myoblasts. β-actin was used as an internal control to ensure equal sample loading.

6.6. Discussion

The alternative splicing factor, hnRNP H, has been found as a dominant binding factor to the CUG repeats in DM1 myoblasts. Since limited amounts of hnRNP H are expressed in differentiated myoblasts (Liu et al., 2001), this essential alternative splicing factor may be totally depleted by expanded CUG repeats. When hnRNP is over-expressed and relieves the limited protein supply in DM1 cells, defective alternative splicing of the insulin receptor can be rescued (Fig. 6 A and B). Furthermore the elevated level of CUGBP in DM1 can be restored to levels seen in normal myoblasts (Fig. 6C). Most significantly, the overexpression of hnRNP H can show a partial rescue of defective alternative splicing even in congenital DM1 myoblasts. This is an important principal, which can be applied to a potential gene therapy for DM1.

Based on this study's data, we present a novel model showing how hnRNP H and RNAs with expanded CUG repeats interact and can be multimerized (Fig. 7). According to the fact that hnRNP H binds to short CUG repeat tracts as a monomer and is not multimerized, stabilization of the CUG hairpin structure may be essential for hnRNP H-mediated multimerization. If the RNA is long enough (such as $(CUG)_{85}$), it can dimerized and further tetramerized in the presence of an unidentified "docking molecule". Recombinant hnRNP H itself can bind to the CUG repeats without any additional factors in vitro (Fig. 4A and 4B). However, a binding kinetic study using the recombinant hnRNP H and muscleblind proteins would explain if the binding of muscleblind to CUG hairpin structure enhances binding of hnRNP H to the RNA. We tested if the muscleblind protein can be the docking molecule. When the recombinant hnRNP H is mixed with recombinant muscleblind protein, there were no signs of multimerization, indicating that muscleblind is not the docking molecule (data not shown). Based on the hnRNP H reconstitution experiment, we can conclude that multimerization of hnRNP H is achieved in two consecutive steps. When the CUG hairpin structure is stable enough to bind hnRNP H, the docking molecules binds

to hnRNP H and causes further multimerization (Fig. 4C). Currently, identifying a docking molecule and determining its functional importance in DM1 pathology is elusive.

Surprisingly enough, the $(CUG)_{129}$ RNA without any extra downstream sequences does not bind to hnRNP H in our native gel assay (Fig. 4D). When $(CUG)_{129}$ RNA is used in a cross linking experiment, we didn't see any significant cross linking products (data not shown). According to serial deletions and mutagenesis, the CUG repeats as well as the splicing branch point in the downstream region are essential for hnRNP H binding. It has been shown that the mutant CUG repeats tract is necessary for nuclear foci formation but not sufficient to disrupt C2C12 myoblast differentiation (Amack and Mahadevan, 2001). RNA foci formation caused by an expanded CUG tract can be separated from the phenotype of myoblast differentiation defect which is mediated by the CUG repeats and proximal sequence of 3-UTR RNA of DMPK. Our study suggest that the CUG repeats as well as the 3' UTR RNA sequence containing the 3' splicing branch point of exon 16 are essential for hnRNP H binding. The involvement of hnRNP H in the nuclear retention of RNA containing CUG repeats has been confirmed by expression of anti-hnRNP H siRNAs in 293 and DM1 cells (Fig. 5C and D). This signifies that the nuclear retention of the (CUG)n RNA is rescued by the interruption of hnRNP H and RNA interaction. Restoration of defective alternative splicing was rescued by overexpression of hnRNP H, but no change in the foci formation was observed (Fig. 6 A and B, and data not shown). This may indicate that the foci formation mediated by the CUG repeats and aggregation of CUG binding factors such as muscleblind may be a partial and not a major determinant of DM1 symptoms.

Overexpression of hnRNP H allows restoration of the alternative-splicing defect of the insulin receptor to normal levels in DM1 myoblasts containing 750 CUG repeats, but only a partial rescue in CDM1 myoblasts (Fig. 6A and B). This may indicate a synergistic effect of hnRNP H and CUG binding factors in determining the pathological phenotype of DM1. If the repeat number is less than 750, the effect of CUG binding factor depletion can be

minimum and the defect in the alternative splicing can be restored by simple supplement of hnRNP H. When the CUG repeat is longer, depletion of a CUG binding factor can be significant and can't be restored to the wild type level by the overexpressed hnRNP H. Combining these studies, the RNA dominant model is attributed by the CUG repeats and other boundary sequence of 3' UTR sequence of DMPK such as the branch point sequence of exon 16. The full repertoire of depleted proteins may not exclusively be limited to hnRNP H or the other CUG binding proteins identified.

The dominant RNA model was demonstrated by transgenic mice expressing an expanded CUG repeat fused to the human actin gene (Mankodi et al., 2000). The mice showed the development of partial DM1 phenotype including myotonia and myopathy. In this study, the splicing branch point was not included. Transgenic mice that have the complete human DM1 locus present a phenotype very similar to that seen in humans (Seznec et al., 2001; Seznec et al., 2000; Gourdon et al., 1997). These mice show the complete repertoire of symptoms including the myotonia, myopathy, histological and electrophysical abnormalities seen in DM1 patients.

The current pathogenesis model for DM1 is explainable by the interaction between the mutant DMPK mRNA and the double-stranded RNA binding protein muscleblind and also by elevated levels of CUGBP in the nucleus (Faustino and Cooper, 2003; Philips et al., 1998; Roberts et al., 1997; Timchenko et al., 1996; Timchenko et al., 2002). Since the function of muscleblind is unknown, it remains to be determined how co-localization of this protein with CUG-repeat RNA could explain splicing defects and DM1 pathogenesis (Fardaei et al., 2002). When CUGBP is overexpressed in normal cells, aberrant splicing patterns of co-trasnfected IR and cTNT minigenes are observed (Philips et al., 1998). A general model for the pathogenic mechanism causing DM1 is currently explained by overexpression of CUG-BP and consequent mis-regulation of splicing of its target pre-mRNAs. The mechanism by which CUG-repeat RNA induces CUGBP expression is still unknown. When the level of hnRNP H is increased in DM1 myoblasts, the aberrant

splicing defect in the IR and the elevated level of CUGBP can be restored (Fig. 6A, B, C). How does overexpression of hnRNP H restore normal levels of CUGBP? Is elevated CUGBP in DM1 directly mediated by depletion of hnRNP H? Further investigation regarding the relationship between CUGBP and hnRNP H will answer these questions and explain the molecular mechanism of DM1.

6.7. Acknowledgments

We thank Dr Black's laboratory for the recombinant hnRNP H clone and anti-hnRNP H antibody and Helfman's lab for the anti-hnRNP H antibody. D. Kim is a Beckman Fellow. M-A. Langlois is a recipient of a doctoral scholarship award from the Canadian Institutes of Health Research. This work was supported by a grant from the Arnold and Mabel Beckman Foundation and the National Institutes of Health (AI29329 and HL074704) and from a grant to J.P. from the Canadian Institutes of Health Research.

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6.9. Figure legends

Figure 1. **UV cross linking of CUG repeat RNAs**. **A)**. RNA substrates used for the UV cross linking experiment. The *SacII/PstI* fragments of DMPK gene with 5, 46, or 85 CTG repeats were cloned and transcribed. **B)** A UV cross linking experiment using the HeLa nuclear extract. Lane 1, cross linked with $(CUG)_5$, lane 2, with $(CUG)_{85}$, lane 3, with $(CUG)_{46}$, lane 4, with biotinylated $(CUG)_{46}$, and lane 5, with biotinylated $(CUG)_{85}$. **C)** UV cross linking using different extracts. Lanes 1 and 2, HeLa total extract, lanes 3 and 4, total DM1 extract before differentiation, lanes 5 and 6, total DM1 extract after differentiation.

Figure 2. **Purification and identification of a novel CUG binding protein**. **A)** Purification of the CUG binding protein. Left panel, brief summary of purification procedure. Right panel, the eluted proteins from the RNA affinity column using $(CUG)_{46}$ or $(CUG)_{85}$ substrate RNAs were separated on a SDS-PAGE gel. **B)** Identification of the protein by mass spectrometry. The sequenced peptide sequences are listed.

Figure 3. **HnRNP H is multimerized to the CUG repeats**. **A)** Identification of the cross linking protein as hnRNP H. The cross linking experiments were done in the absence (lanes 1 and 2) or presence of the anti-hnRNP H antibody (lanes 3 and 4). **B)** The hnRNP H antibody can immuno-purify the 50 and 100 Kda cross-linking products. **C)** The CUG repeats and hnRNP H forms a multi-complex in a native gel. Lanes 1 and 2, the $(CAG)_{85}$ RNA with or without the total extract. Lanes 3, $(CUG)_{85}$ RNA itself, lane 4, $(CUG)_{85}$ with the total extract, lanes 5 and 6, $(CUG)_{85}$ with the total extract pre-incubated with the

preimmune serum. Lanes 7 and 8, $(CUG)_{85}$ with the total extract pre-incubated with the hnRNP H antiserum.

Figure 4. The recombinant hnRNP H doesn't multimerized. **A)** The cross linking assay was done using total cell extracts (lanes 1 and 2) or the recombinant hnRNP H protein (lanes 3 and 4). **B)** The recombinant hnRNP H binds to the CUG repeats but doesn't form a multi-complex. Lanes 1, $(CUG)_5$ only, lane 2, $(CUG)_5$ with the total extract, lane 3, $(CUG)_5$ with the 1 ng of recombinant hnRNP H. Lane 4, $(CUG)_{46}$ with the total extract, lane 5, $(CUG)_{46}$ with the total extract, lane 6, $(CUG)_{46}$ with the 1 ng of recombinant hnRNP H. Lane 7, $(CUG)_{85}$ itself, lane 8, $(CUG)_{85}$ with the total extract, lane 9, $(CUG)_{85}$ with 1 ng of the recombinant hnRNP H. **C)** A cellular factor is required for hnRNP H to be multimerized. Lane 1, $(CUG)_{85}$ only, lane 2, the RNA with the total HeLa extract, lane 3, the RNA with the hnRNP H depleted HeLa extract. Lane 4, the RNA with 10 ng of recombinant hnRNP H, lane 5, the RNA with the reconstituted extract, which was mixed and incubated the depleted extract and 10 ng of purified hnRNP H before the RNA binding reaction. To confirm the immunodepletion of hnRNP H, total amount of hnRNP H was compared before (lane 2) and after depletion (lane 3). **D)** The hnRNP H binding requires the CUG repeats and the branch point of exon 16 located the distal region of the repeated sequence. The right panel indicated each RNA clones created in these experiments. RNA #1 has the sequence of the proximal sequence, CUG repeats and the splicing branch point, RNA #2 has the same sequence except the mutated branch point. RNA#3 has the CUG repeats and the branch point. RNA#4 has 129 repeats of CUGs. The left panel indicates a binding assay on the native gel. Lane 1, #1 RNA itself, lane 2, #2 RNA with 10 ng of hnRNP H, lane 3, #1 RNA with 10 ng of hnRNP H, lane 4, #3 RNA with 10 ng of hnRNP

H, lane 5, RNA #1 with total extract. Lanes 6 and 7, RNA #4 with or without 10 ng of hnRNP H.

Figure 5. **The expression of hnRNP H is reduced in DM1 myoblasts A)** The expression level of hnRNP H was compared to 293 (lane 1), HeLa (lane 2), and normal myoblasts (lanes 3 and 4) or DM1 myoblasts (lanes 5 and 6) before (lanes 3 and 5) or after differentiation (lanes 4 and 6) by Western blot. The amount of total proteins in each lane was adjusted by protein staining (data not shown). **B)** In situ hybridization of hnRNP H level before and after differentiation of DM1 cells. **C)** Anti-hnRNP H siRNAs reduce the nuclear retention of eGFP-CUG repeats reporter gene. When the 293 cells are transfected with an eGFP reporter gene with the $(CUG)_5$, normal eGFP expression was observed (panel 1). If the cells are transfected with the eGFP-CUG85 clone and an irrelevant siRNA, a reduction of eGFP expression is observed (panel 2) because nuclear retention of the RNA as described elsewhere (Amack and Mahadevan, 2001). When the reporter gene is co-transfected with the anti-hnRNP H siRNAs (panel 3 and 4), eGFP expression is restored. **D)** Anti-hnRNP siRNA can restore the reduced expression of the eGFP reporter gene in DM1 cells. **E)** HnRNP H and CUG repeats interact in vivo. Normal myoblasts were transfected with the body labeled CUG repeats and cross linked by UV irradiation. HnRNP H in the total extract was immunopurified using the hnRNP H antibody. HnRNP H associated RNAs were extracted and resolved on a denaturing gel, a northern blot was the performed using a labeled CAG10 probe.

Figure 6. Overexpression of hnRNP H restores alternative splicing of the IR mRNA. A) A reporter gene of eGFP (lanes 1 and 3) or hnRNP H-eGFP fusion gene (lanes 2 and 4)

was cloned under the CMV promoter and transfected into normal (lanes 1 and 2) or DM1 myoblasts with 750 CTG repeats (lanes 3 and 4). Total RNA was prepared from eGFP positive cells and used for RT-PCR to determine the ratios of isoforms A and B of the insulin receptor. **B)** The parallel experiment was performed using congenital DM1 myoblasts with 3000 CTG repeats. **C)** Overexpression of hnRNP H restores the elevated level of CUGBP expression in DM1 cells. Lane 1, from the wild type cell, lane 2, from the DM1 cells with the vector transfected, lane 3, from the DM1 cells with overexpression of hnRNP H.

Figure 7. A proposed model for the binding and multimerization hnRNP H to the CUG repeats. HnRNP H binds to the stabilized CUG hairpin structure and the branch point sequence (E16bp). By association with an unidentified docking protein (DP), the RNA/hnRNP H complex can form dimers or tetramers.

6.10. Figures

FIGURE 1

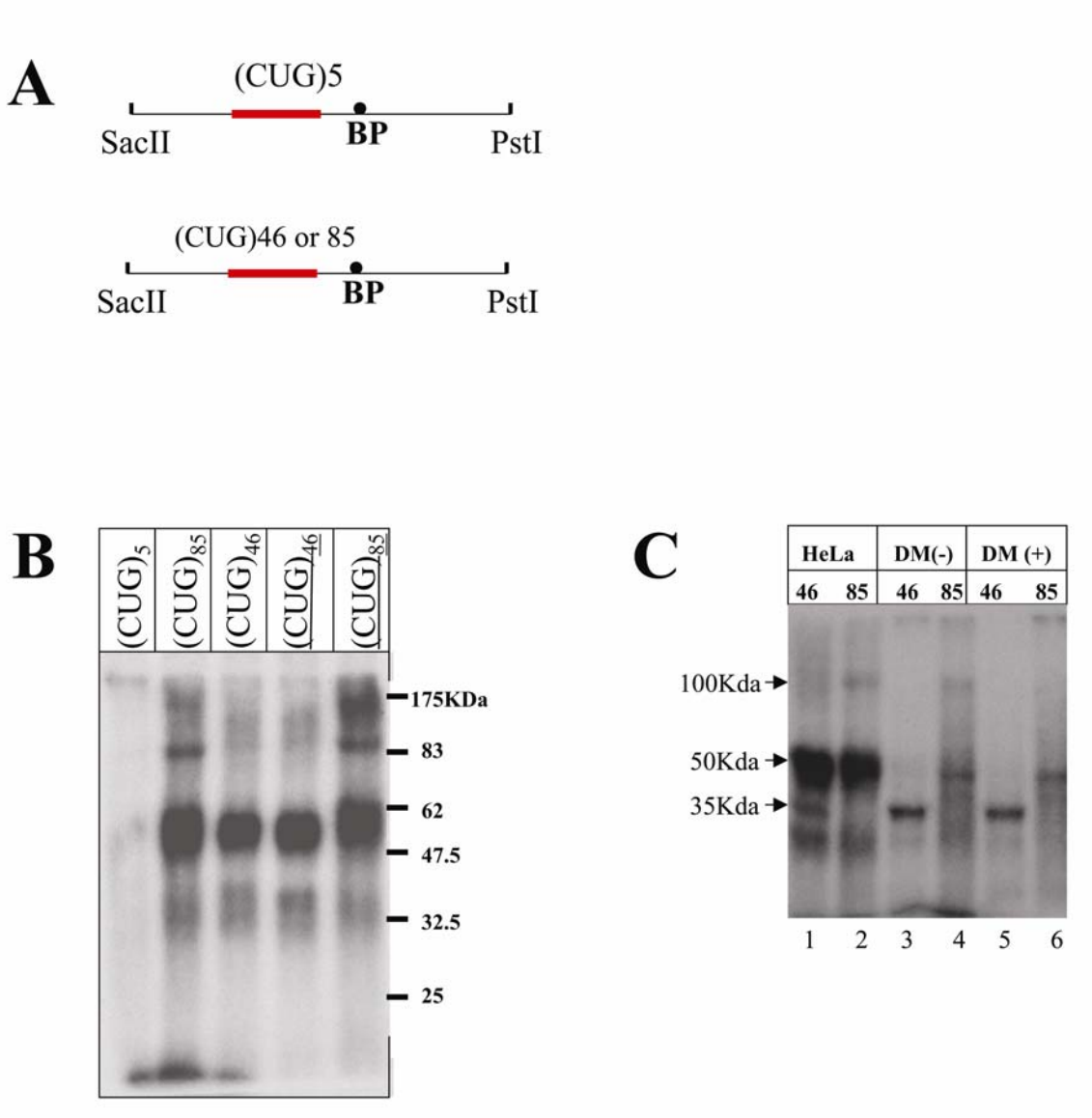
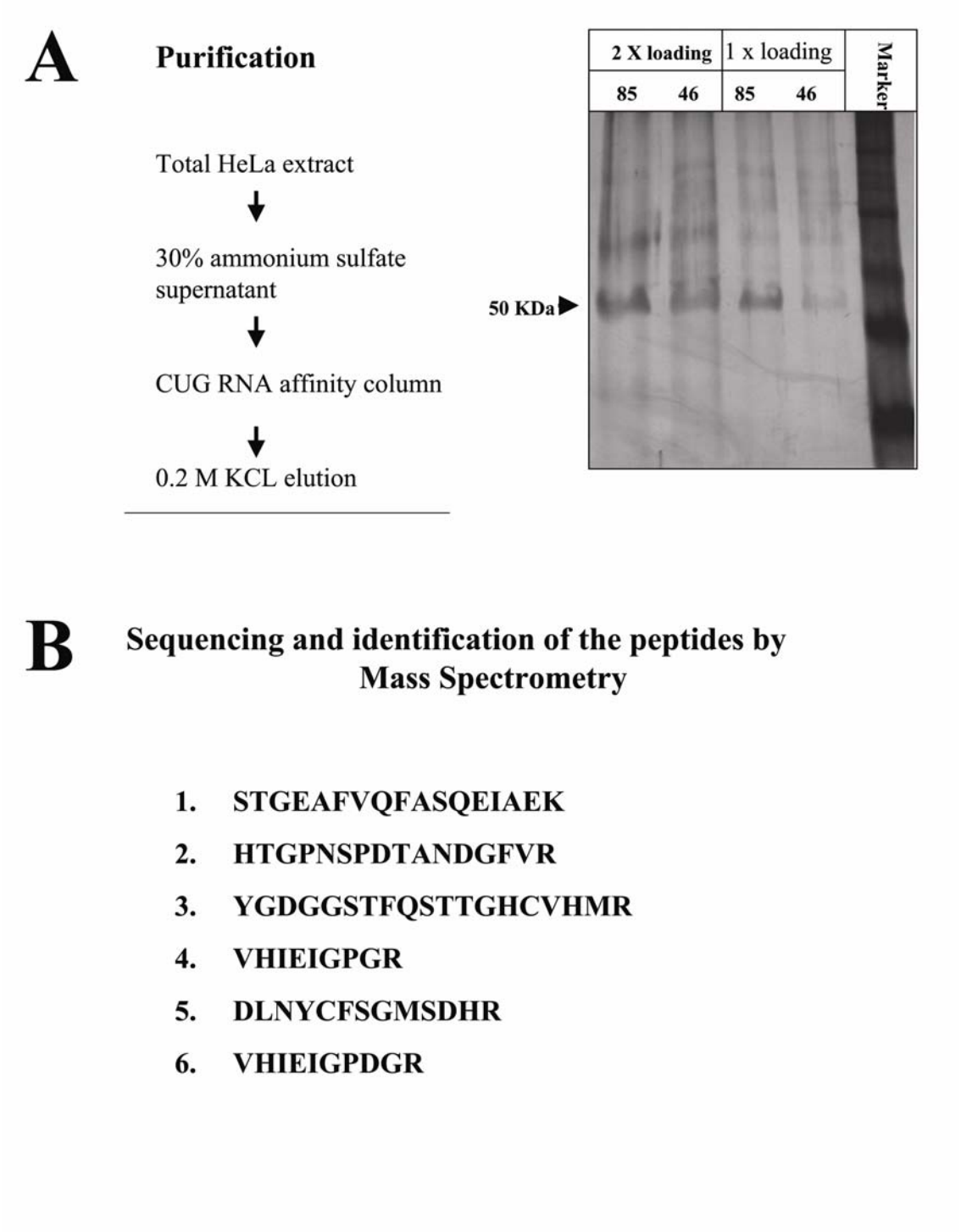


FIGURE 2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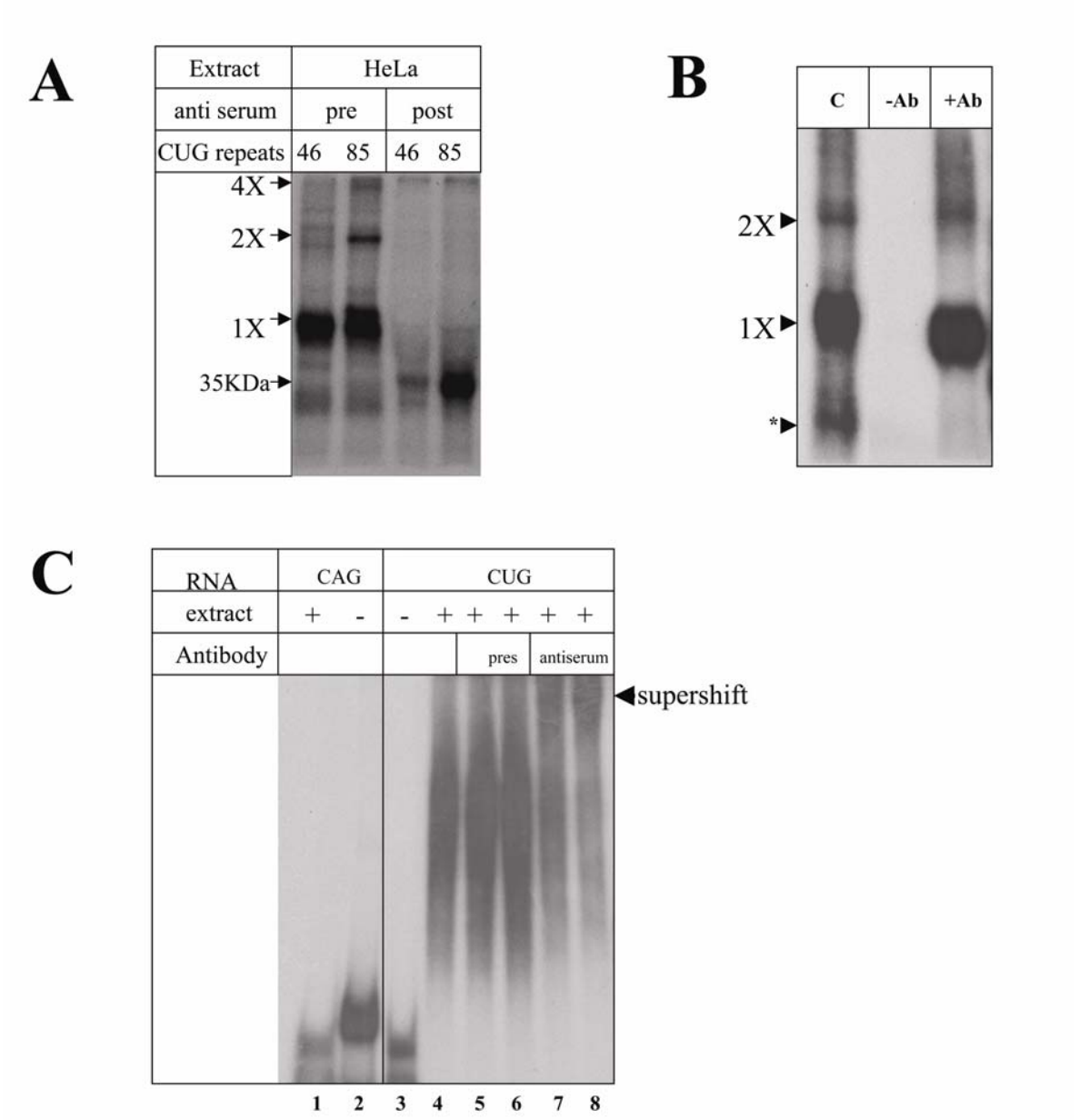
Purification
Total HeLa extract
30% ammonium sulfate supernatant
CUG RNA affinity column
0.2 M KCL elution
2 X loading
1 x loading
Marker
85
46
85
46
50 KDa
B
Sequencing and identification of the peptides by Mass Spectrometry
1. STGEAFVQFASQEIAEK
2. HTGPNSPDTANDGFVR
3. YGDGGSTFQSTTGHCVHMR
4. VHIEIGPGR
5. DLNYCFSGMSDHR
6. VHIEIGPDGR

FIGURE 3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Extract
HeLa
anti serum
pre
post
CUG repeats
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85
46
85
4X
2X
1X
35KDa
B
C
-Ab
+Ab
2X
1X
*
C
RNA
CAG
CUG
extract
+
-
-
+
+
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Antibody
pres
antiserum
supershift
1
2
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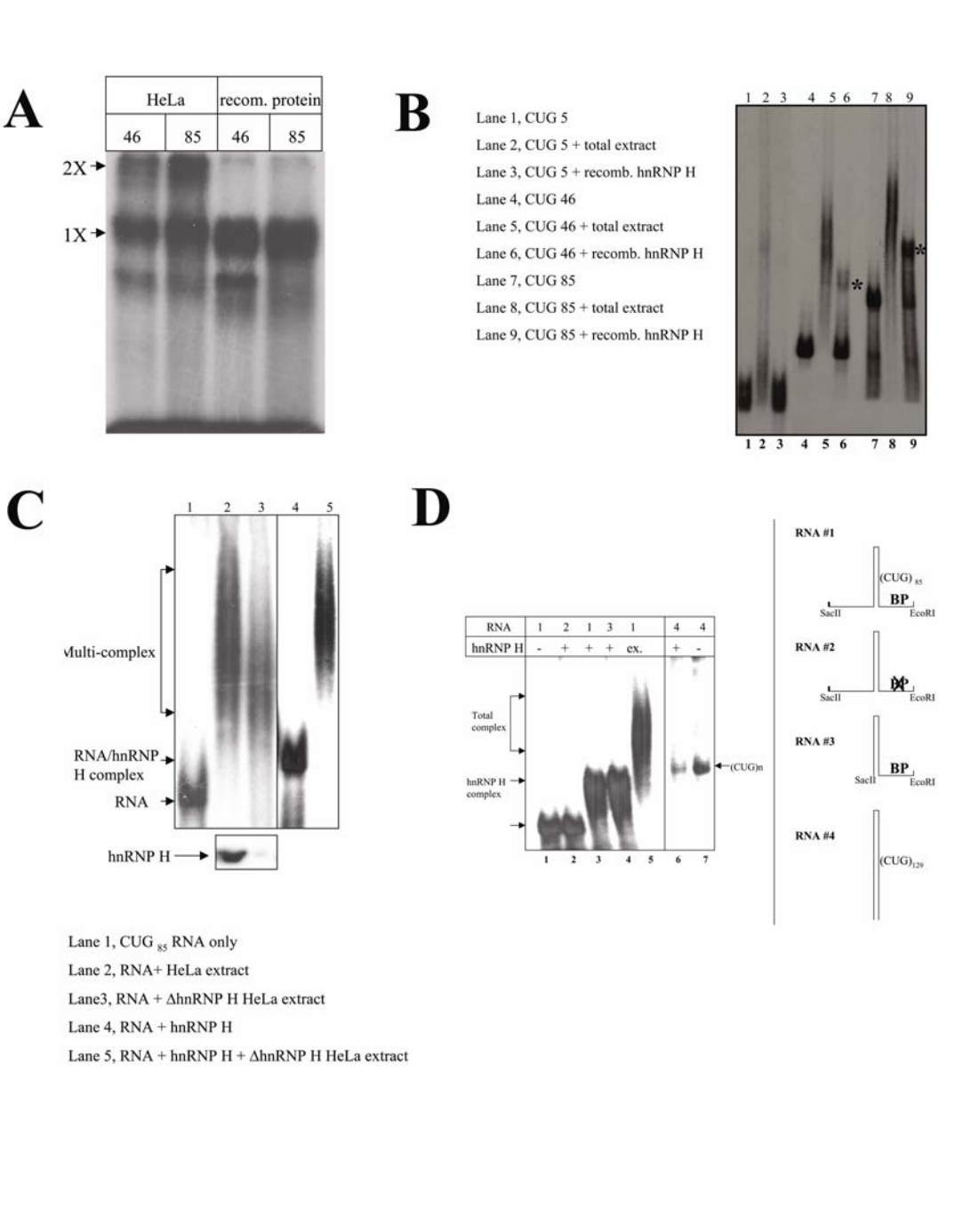
7
8

FIGURE 4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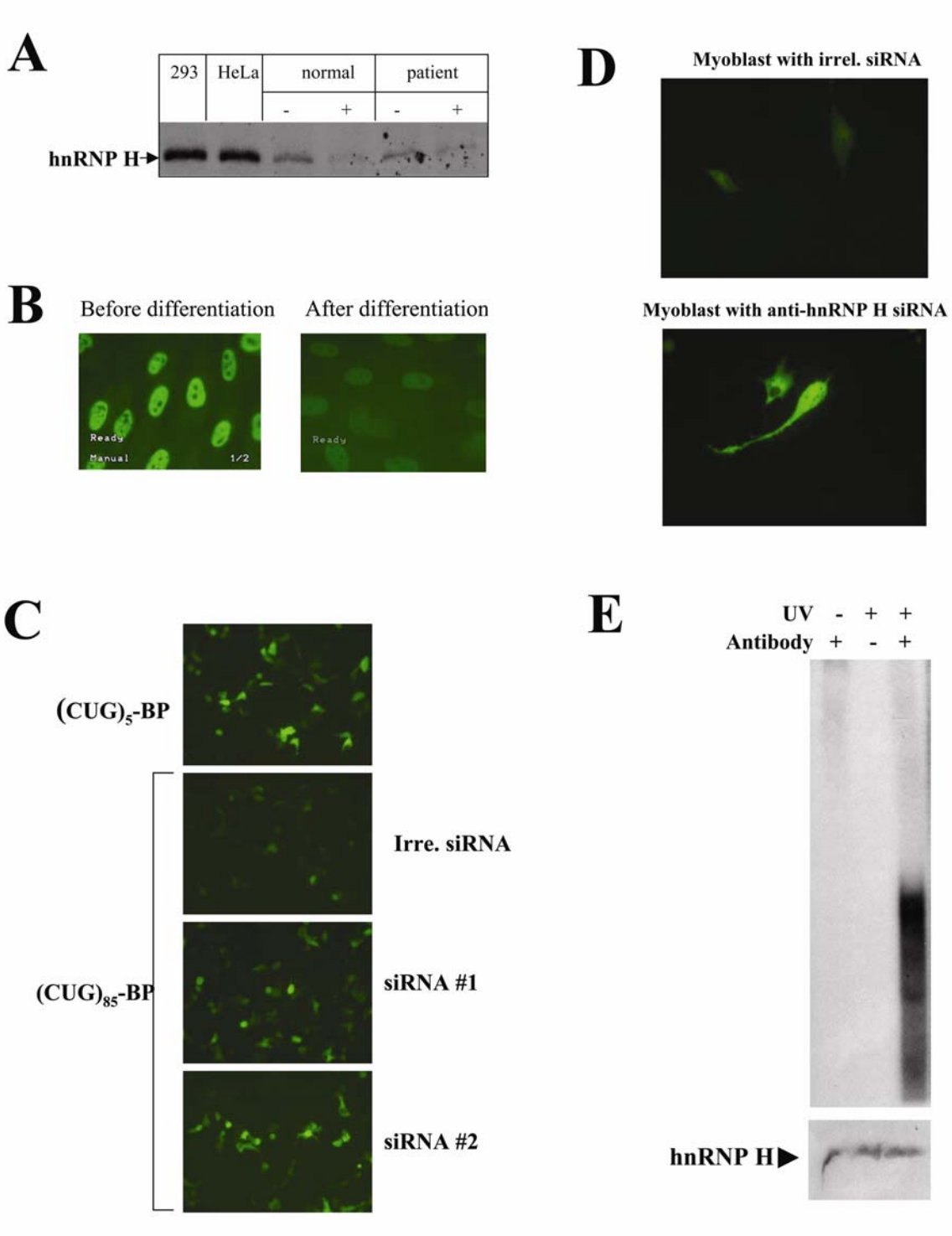
HeLa
recom. protein
46
85
46
85
2X
1X
B
Lane 1, CUG 5
Lane 2, CUG 5 + total extract
Lane 3, CUG 5 + recomb. hnRNP H
Lane 4, CUG 46
Lane 5, CUG 46 + total extract
Lane 6, CUG 46 + recomb. hnRNP H
Lane 7, CUG 85
Lane 8, CUG 85 + total extract
Lane 9, CUG 85 + recomb. hnRNP H
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5
Multi-complex
RNA/hnRNP H complex
RNA
hnRNP H
D
RNA 1 2 1 3 1 4 4
hnRNP H - + + + ex. + -
Total complex
hnRNP H complex
(CUG)n
1 2 3 4 5 6 7
RNA #1
(CUG) 85
BP
SacII
EcoRI
RNA #2
RNA #3
RNA #4
(CUG)129
Lane 1, CUG 85 RNA only
Lane 2, RNA+ HeLa extract
Lane3, RNA + ΔhnRNP H HeLa extract
Lane 4, RNA + hnRNP H
Lane 5, RNA + hnRNP H + ΔhnRNP H HeLa extract

FIGURE 5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293
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patient
-
+
-
+
hnRNP H
B
Before differentiation
After differentiation
Ready
Manual
1/2
Ready
C
(CUG)5-BP
Irre. siRNA
(CUG)85-BP
siRNA #1
siRNA #2
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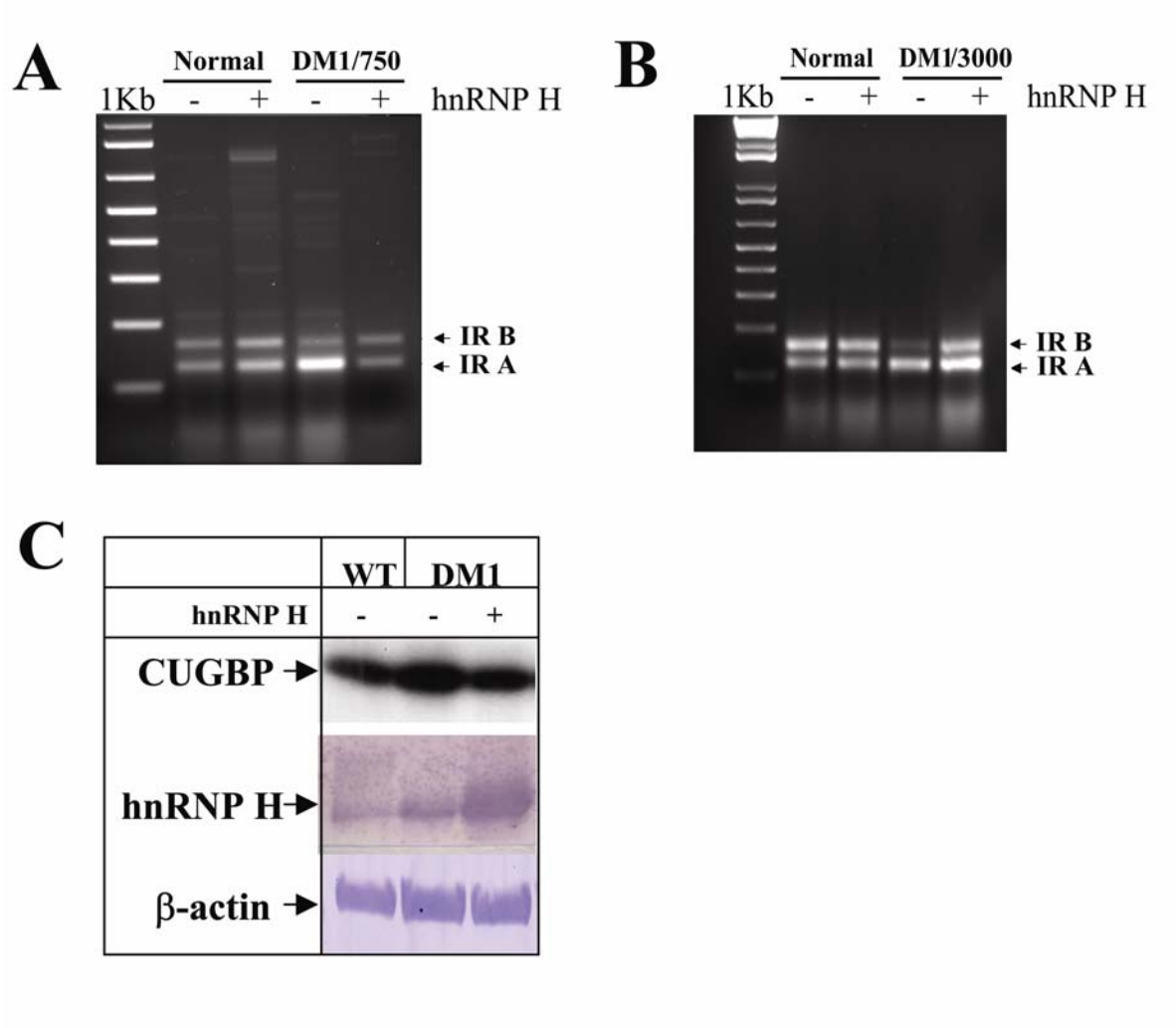
Myoblast with irrel. siRNA
Myoblast with anti-hnRNP H siRNA
E
UV - + +
Antibody + - +
hnRNP H

FIGURE 6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-
+
-
+
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IR B
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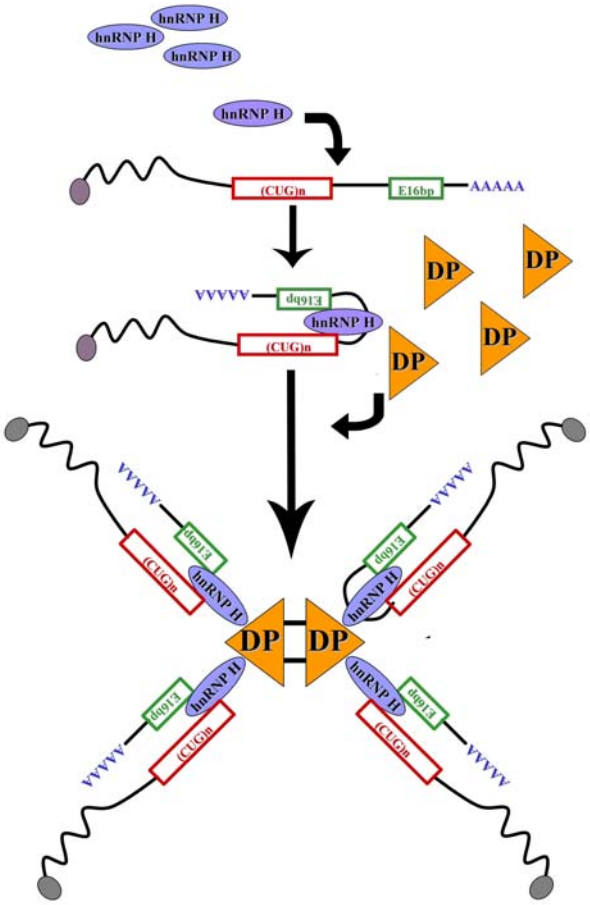
B
Normal
DM1/3000
1Kb
-
+
-
+
hnRNP H
IR B
IR A
C
WT
DM1
hnRNP H
-
-
+
CUGBP
hnRNP H
β-actin

FIGURE 7


hnRNP H
hnRNP H
hnRNP H
hnRNP H
(CUG)n
E16bp
AAAAA
DP
DP
DP
DP
VVVVV
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(CUG)n
VVVVV
E16bp
hnRNP H
(CUG)n
VVVVV
E16bp
hnRNP H
(CUG)n
DP
DP
hnRNP H
E16bp
(CUG)n
VVVVV
hnRNP H
E16bp
(CUG)n
VVVVV

GENERAL CONCLUSIONS

The work presented in this thesis can be divided in three parts: development of molecular tools and of a cell culture model, validation of RNA-based therapeutic approaches and identification of a novel CUG-binding protein with an important role in myotonic dystrophy type 1. In chapter II, we laid-down the foundation for standard experimental conditions that were used in all subsequent work. We established an effective and rapid method for completely extracting mutant DMPK mRNA. Improper extractions have led to a vast number of ambiguous and contradicting reports on mutant DMPK levels. We also set a standardised method for Northern blotting, allowing consistency and reproducibility. Furthermore, we optimized conditions for human skeletal myoblast culture growth and differentiation in our laboratory. Differences in cell culture conditions may lead to delays in myogenic marker expression and may alter kinetics of DMPK mRNA and protein expression. Since the symptoms of adult onset and congenital myotonic dystrophy can be relatively different, it was imperative that we characterized our stocks of myoblasts since most of them come from myotonic dystrophy fetuses without knowing if they had the congenital form of the disease or not.

In the second part of our work we employed antisense RNAs, ribozymes and siRNAs to answer one specific question: can the normal phenotype be restored in myotonic dystrophy myoblasts by destroying mutant DMPK mRNA? The answer is yes, but with some nuances. First, there is a considerable difference between studying a phenotype in cultured cells than in cells that form living tissue. There are many dynamic features in living tissue that cannot be successfully reproduced or measured *in vitro*, myotonia and muscle weakness are two examples. Next, although some features of the disease phenotype were restored in skeletal muscle myoblasts, it is still uncertain that defects in lens cells, neurones or cardiac muscle will show similar results. However, results obtained are very encouraging and there is no reason to believe that these therapeutic approaches aimed at destroying mutant DMPK

mRNA should not prove successful in cells from other tissues, if the RNA dominance model for myotonic dystrophy is respected.

Antisense RNAs were shown successful in restoring myoblast fusion, glucose uptake and also in reducing the levels of nuclear CUGBP (Chapter III). Although the exact mode of action of long antisense RNAs is still unclear, it is likely that the AS-RNAs were retained in the nucleus of the myoblasts since eIF-2α was not phosphorylated, protein synthesis was not altered and no apotosis was observed in our transduced myoblasts (data not shown). These cardinal features of an interferon response would have been triggered if the long AS-RNAs had reached the cytoplasm. A likely explanation for these results could be that a stable duplex forms between mutant DMPK mRNA and the AS-RNA producing a substrate for ADARs, but also for RNAse-III-like enzyme activity in the nucleus. In addition, as we have shown in chapter V, there exist molecular components of the RNAi pathway in the nucleus of myoblasts. It could well be possible that long AS-RNAs are processed into siRNAs and induce PTGS in the nucleus. Cleavage activity resulted in nearly 80% destruction of mutant DMPK mRNA. It is not surprising that glucose uptake was restored in AS-transduced myoblasts since nuclear CUGBP levels were restored. As seen in chapter VI, reduction of CUGBP in the nucleus has a direct impact on alternative splicing defects. With lowered levels of CUGBP in these myoblasts, it seems obvious that the insulin receptor alternative splicing defect was to be restored. This however, needs to be verified experimentally.

Ribozymes were also very effective in reducing mutant DMPK mRNA levels in the nucleus (Chapter IV). These ribozymes were expressed by a modified tRNAmeti promoter that allows nuclear retention. TRNAmeti-Ribozymes preferentially targeted mutant transcripts, although a certain number of normal transcripts were also destroyed. As with AS-RNAs, maintaining a minimal level of DMPK protein may be necessary if these molecules are to be eventually used for gene therapy in humans. The main reason is that the exact physiological role for DMPK in humans has not yet been determined. It would thus be

unethical to silence expression of a gene whose function is not well defined. What we observed in these myoblasts expressing the ribozymes was a reduction in the number and the intensity of nuclear foci. This suggests that the CUG repeat tracts were dissolved and nuclear factors bound to them liberated. Correction of the insulin receptor mRNA alternative splicing is another proof that essential splicing factors that bind to the expanded CUG repeats were likely made available for normal cellular functions.

The third RNA-based approach tested was the use of shRNAs to induce RNAi (Chapter V). Initially, reports stated that RNAi was exclusively a cytoplasmic phenomena (Kawasaki and Taira, 2003; Zeng and Cullen, 2002). When we first started this project, part of our goal was to validate these results, but also to produce myoblasts expressing only mutant DMPK mRNA. This would have allowed us to study the consequences of complete knockdown of DMPK protein expression. To our surprise, strong RNAi activity was detected in the nucleus of myoblasts. This activity is RNAse-driven since gene transcription rates were similar to controls. However, shRNAs are less adequate than ribozymes and AS-RNAs for use in a gene therapy for myotonic dystrophy because they do not discriminate between normal and mutant DMPK mRNAs. Our results have also shown that they work almost 20% better in the cytoplasm. ShRNAs would reveal themselves as very powerful tools in myotonic dystrophy research if they could achieve complete silencing of both normal and mutant DMPK mRNAs.

Although these three methods have successfully reduced the levels of mutant DMPK mRNA and have restored several parameters of a normal phenotype in myotonic dystrophy myoblasts, it is still unclear what effects will result from lowered DMPK protein levels *in vivo*. Electrophysiological studies showed that DMPK -/- mice develop cardiac conduction defects similar to those seen in myotonic dystrophy patients (Berul et al., 1999). Future projects need to address whether downregulation of DMPK protein using our methods induce these disruptions in mice and also determine what is the minimal level of protein necessary for normal cardiac function.

Another interesting issue that needs to be addressed is vector delivery. These three RNA-based approaches all used different delivery systems: the AS-RNAs were delivered by oncoretroviruses, the ribozymes were transiently expressed following transfection with cationic lipids and the shRNAs were stably expressed following lentivirus transductions. Because it is our plan to test these molecules in mice, it will be necessary to utilize a delivery system that can infect both dividing and differentiated cells. It is also important when transducing dividing cells that the transgene integrates to the genome or else the cells will eventually loose epigenic plasmids through successive divisions. The lentiviral system used to express shRNAs is at the moment the best candidate delivery system to pursue future work since it has all these necessary features and their use has been validated in skeletal muscle (Federico, 1999; MacKenzie et al., 2002; Naldini, 1998; Sakoda et al., 1999; Seppen et al., 2001).

Alongside the issues of using the most effective delivery system comes the choice of an appropriate promoter. The ideal promoter system for myotonic dystrophy would allow tissue-specific expression of the therapeutic RNA in the nucleus and even better, in the foci. This localization would ensure a high concentration of therapeutic molecules at the site where mutant DMPK mRNAs accumulate. The nuclear compartment in which the foci form has not been identified yet, and there is now much speculation that the foci themselves constitute a novel pathogenic nuclear compartment, invoking that localization to the foci may not be feasible. To this effect, the choice of the promoter system will depend largely on the therapeutic RNA we want to express. ShRNAs for example have strict sequence requirements and Pol II tissue-specific promoters may not be the right choice for these molecules. Ribozymes and long antisense RNAs on the other hand can be readily expressed by a muscle specific promoter such as MCK for example. This would insure that expression would occur only in skeletal muscle which is the most heavily affected tissue in myotonic dystrophy.

In the third and final part of our work, we were interested in identifying a novel CUG-binding protein. Muscleblind (MBNL) are the only proteins to date that co-localize with the foci in myotonic dystrophy cells. Aside from these proteins having a role in muscle and photoreceptor cell development in Drosophila, their implication in myotonic dystrophy is still elusive. We have demonstrated that hnRNP H binds specifically to expanded CUG repeats when the cryptic exon 16 branch point is located downstream from the repeats. HnRNP H can form dimers and tetramers with the aid of an unidentified docking protein. Our data suggests that these multimeric structures are responsible for retaining mutant DMPK mRNA in the nucleus. Furthermore, overexpression of hnRNP H reduces nuclear levels of CUGBP. This may explain why insulin receptor splicing was also restored by hnRNP H overexpression. Seemingly, there is a direct relationship between the presence of CUG repeats and CUGBP expression in myotonic dystrophy. In the antisense study, CUGBP levels were restored when mutant DMPK mRNA was destroyed. In chapter VI we showed that CUGBP levels are restored when hnRNP H is overexpressed. It now seems obvious that nuclear depletion of hnRNP H through binding to the repeat tracts is directly involved in modulating CUGBP nuclear levels. Interestingly, when hnRNP H is over expressed, foci are still present. This suggests that splicing defects are foci-independent but correlate directly with CUGBP nuclear levels.

Other proteins that may or may not have roles in myotonic dystrophy pathogenesis are also present in these foci. Depletion of these proteins could cause other alterations such as muscle weakness and myoblast fusion defects. It is now urgent to determine whether cell fusion is restored when hnRNP H is overexpressed. Since destruction of mutant DMPK mRNA correlates with restoration of cell fusion, it confirms that the protein(s) responsible for this defect is (are) associated with the mutant DMPK mRNA. Whether hnRNP H is responsible for foci formation or for the vast ensemble of myotonic dystrophy symptoms is still a moot point that needs to be addressed.

The sum of these results urges us now to review the current model for myotonic dystrophy pathogenesis. Figure 18 illustrates a revised model that explains the relationship between foci formation, mutant DMPK mRNA nuclear retention, and aberrant alternative splicing.

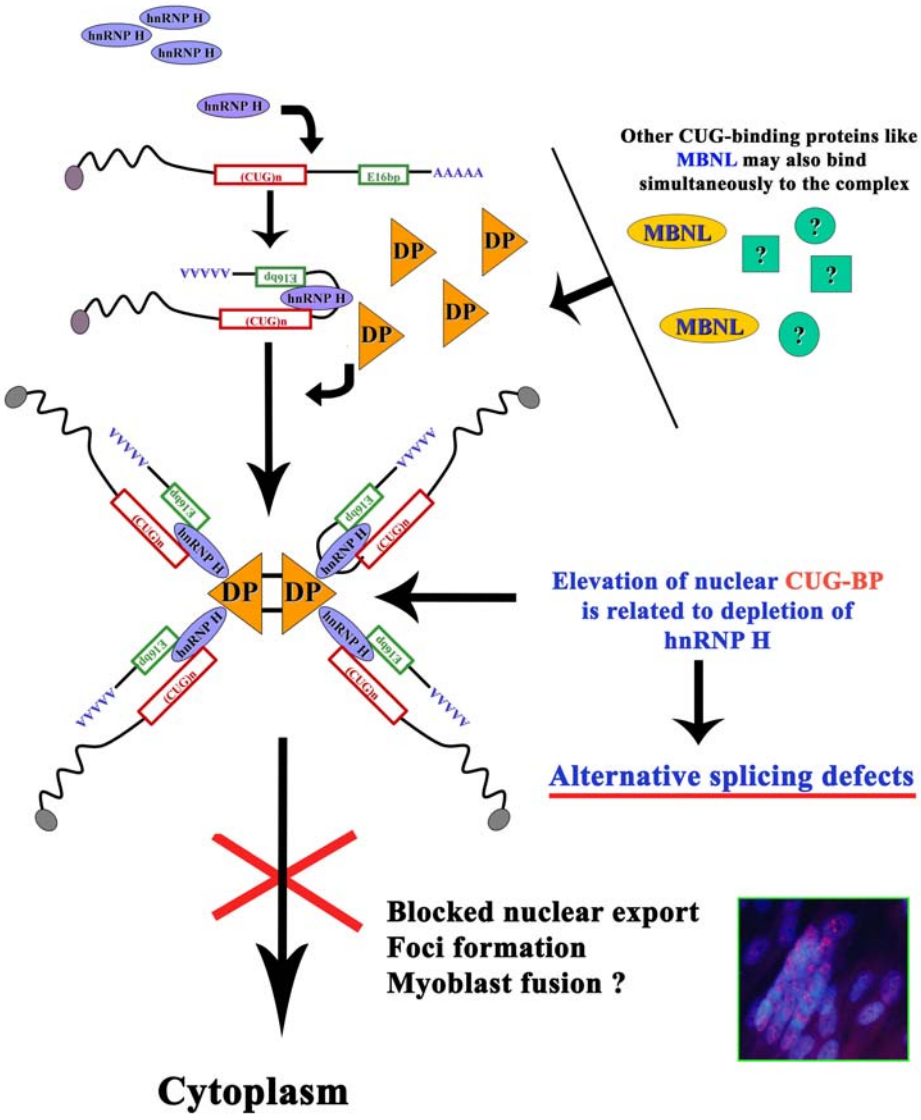

Figure 18 Revised molecular pathogenesis model for myotonic dystrophy type 1

HnRNP H has weak affinity for both CUG-repeats ***((CUG)n)*** *or the exon 16 branch point* ***(E16bp)*** *located in 3'UTR of the mutant DMPK mRNA. When both binding sites are*

present, hnRNP H stably binds and can be detected by UV-cross linking experiments. A docking protein ***(DP)*** *can in addition allow formatiom of complexes with two or four mRNA molecules. These large complexes are incapable of being exported and form foci in the nucleus. Levels of hnRNP H are very scarce during myoblast differentiation. Binding to CUG-repeats will likely deplete hnRNP H and induce the expression of nuclear CUGBP through an unknown mechanism. Nuclear CUGBP accumulation is related to aberrant alternative splicing of several mRNAs. Although MBNL proteins co-localize with the nuclear foci, they are not implicated in hnRNP H binding. Their involvement in myotonic dystrophy is still unknown.*

Our work has established for the first time that it is possible to target mutant DMPK mRNA in skeletal muscle myoblasts and reduction of these expression levels coincides with the rescue of several molecular defects characteristic of myotonic dystrophy. It is unclear at this time whether such an approach will be necessary in view of developing an eventual gene therapy, due to restoration of alternative splicing defects obtained from hnRNP H overexpression. Restoration of the nuclear levels of sequestered proteins may prove to be more effective and simpler than destroying mutant DMPK mRNA. Subsequently, it will be important to assess the pros and cons of a gene therapy versus a cell therapy whereby stem cells are genetically modified *in vitro* and transplanted *in vivo* where they differentiate into healthy muscle. To this effect, it is now necessary to take this work to the next logical step and validate our results in a myotonic dystrophy type 1 mouse model using lentiviral vectors for delivery. Then only can we assess the true feasibility of developing a treatment for myotonic dystrophy the in the near future.

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APPENDIX 1

Intracellular ribozyme applications

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Abbreviations used: DMPK, dystrophica myotonica-protein kinase ; MDM2, murine double minute 2.

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Résumé

La grande spécificité des ribozymes agissant en *trans* laisse entrevoir l'énorme potentiel de ces ARNs en thérapie génique. Il est essentiel afin de mettre au point un ribozyme efficace de co-localiser l'ARN cible et le ribozyme. De plus, il est nécessaire d'identifier des sites sur l'ARN cible qui sont dénudés de protéines pour ainsi permettre la liaison du ribozyme. Cet article discute de la mise-au-point de ribozymes efficaces, des systèmes permettant leur expression et une localisation cellulaire spécifique, ainsi que de leurs applications potentielles en thérapie.

Abstract

The exquisite target selectivity of trans -acting ribozymes has fostered their use as potential therapeutic agents and tools for down-regulating cellular transcripts. In living cells, free diffusion of RNAs is extremely limited, if it exists at all. Thus, getting ribozymes to base-pair with their cognate targets requires co-localizing the ribozyme transcript with the target RNA. In addition, not all sites along a given target RNA are equally accessible to ribozyme base pairing. Cellular proteins greatly influence the trafficking and structure of RNA, and therefore making ribozymes work effectively in cells a significant challenge. This article addresses the problems of getting engineered ribozymes to effectively pair with and cleave targets in cells. The work described here illuminates methods for target-site selection on native mRNAs, methods for ribozyme expression, and strategies for obtaining a discrete intracellular localization of ribozymes.

Key words: cancer, functional genomics, genetic disease, HIV, nucleolus.

Introduction

Ribozymes are RNA molecules that are capable of acting as enzymes, even in the complete absence of proteins. They have the catalytic activity of breaking and/or forming covalent bonds with extraordinary specificity, thereby accelerating the rate of these reactions. The ability of RNA to serve as a catalyst was first shown for the self-splicing Group I intron of Tetrahymena and the RNA component of ribonuclease P [1, 2]. Ribozymes occur naturally, but can also be artificially engineered and synthesized to target specific sequences in cis or trans. Ribozymes have been applied as antiviral agents, for the treatment of cancer and genetic disorders, and as tools for pathway elucidation and target validation.

Results and discussion

Initial uses of ribozymes focused on their uses as antiviral agents, primarily for the treatment of HIV [3]. Viruses that have a genomic RNA intermediate in their replication cycle, such as HIV, hepatitis B virus and hepatitis C virus, are at- tractive targets because a single species of ribozyme can target both viral genomic RNA and mRNAs. Ribozymes have also been widely used to target cellular genes, including those that are expressed aberrantly in cancers [4].

We have designed effective ribozymes that target the MDM2 (murine double minute 2) transcript, whose levels are elevated in some cancers. Overexpression of MDM2 blocks the function of p53, resulting in a transformed phenotype. We demonstrated that ribozyme-mediated cleavage of MDM2 transcripts in a cell line that over-expresses this protein results in reduced cellular proliferation and apoptosis (J. R. Li, J. Momand and J. J. Rossi, unpublished work; Figure 1). Thus, MDM2 is a potential therapeutic target for ribozyme-mediated down-regulation in a number of cancers.

In addition to directly targeting oncogenes, ribozymes have also been applied more indirectly in anti-cancer therapy. For example, ribozymes that target the multiple drug resistance-1 [5, 6] or fos [7] mRNAs in cancer cell lines effectively made the cells more sensitive to chemotherapeutic agents. Alternatively, a ribozyme that targets bcl-2 triggered apoptosis in oral cancer cells [8]. Factors that are required for metastasis are also attractive targets for ribozymes. Ribozymes targeted against CAPL (calcium placental protein)/mts (metastasin) [9], matrix metalloproteinase-9 [10], pleiotrophin [11] and VLA-6 integrin [12] all reduced the metastatic potential of the respective tumour cells. Angiogenesis is also an important target of cancer therapy, and has been blocked in mice by ribozymes that target fibroblast growth factor-binding protein [11] and pleiotrophin [13].

Heritable and spontaneous genetic disorders represent additional applications for therapeutic ribozymes that target cellular genes. These include the b-amyloid peptide precursor mRNA, which is involved in Alzheimer's disease [14, 15], and an autosomal-dominant point mutation in the rhodopsin mRNA that gives rise to photoreceptor degeneration and retinitis pigmentosa [16]. A somewhat different type of genetic disorder is associated with the expansion of triplet sequences in diseases such as Huntington's disease and myotonic dystrophy. In myotonic dystrophy, CUG repeats in the 3′ untranslated region can expand into several thousand tandem copies. This expansion results in retention of the DMPK (dystrophica myotonica-protein kinase) mRNA in the cell nucleus, where it forms foci that titrate RNA-binding proteins. We have demonstrated that cleavage of the transcripts with a ribozyme targeted just upstream of the repeats results in degradation of the CUG-repeat-containing mRNAs (Figure 2) and concomitant loss of foci (M.-A. Langlois, N. S. Lee, J. J. Rossi and J. Puyrimat, unpublished work). It is therefore a distinct possibility that a ribozyme therapy for myotonic dystrophy can be developed with the appropriate delivery system for the ribozyme or ribozyme genes.

Optimizing intracellular function of ribozymes

In contrast with the rather extensive knowledge of the rules governing ribozyme function in vitro, where free diffusion of ribozyme and target in solution are unrestricted, there are only a limited set of rules for predicting ribozyme efficacy in a complex intracellular environment. The parameters that affect ribozyme function in cells are intracellular stability, expression levels of the ribozyme RNA, intracellular co-localization with the target RNA, stability of the ribozyme transcripts, and interactions of proteins with the ribozymes. The most effective strategies for achieving ribozyme function in vivo involve mechanisms for maximizing the ability of the ribozyme to pair with its target RNA. Since the trans-cleaving and trans-ligation applications of ribozymes involve Watson–Crick base pairing of the ribozyme, or the guide sequence (for ribonuclease P), to the target RNA, this interaction is the rate-limiting step in vivo . Various strategies have been used to identify

accessible pairing sites on target RNAs. Not all target sites for these ribozymes are accessible for cleavage: secondary structures, the binding of proteins and nucleic acids, and other factors influence intracellular ribozyme efficacy. Computer-assisted RNA-folding predictions and in vitro cleavage analyses are not necessarily predictive for intracellular or in vivo activity, and the best ribozyme target sites often must be determined empirically in vivo. Strategies that use cell extracts with native mRNAs have proven useful for determining accessible ribozyme-binding sites [17–19]. Expression levels and intracellular localization of the ribozyme transcripts are critical for the successful application of the ribozymes in gene therapy. For transcripts, various promoters can be used to obtain either constitutive or regulated expression [20–22]. The transcripts can be engineered to localize within the same cellular or sub-cellular compartment as the target RNA. In order for trans-cleaving ribozymes to be effective in down-regulating mRNAs, the efficiency of cleavage must be greater than the steady-state rates of synthesis and decay of the target.

For ribozyme-mediated down-regulation to be effective, at a given ribozyme concentration the decay mediated by the ribozyme must exceed the steady-state level of target turnover in the absence of the ribozyme. This can be best achieved by identifying highly accessible target sites on the target, combined with high levels of ribozyme expression in the appropriate cellular compartment; using these parameters, down-regulation can exceed 90%.

A combination of the above elements has been applied to an anti-HIV-1 ribozyme. A ribozyme targeted to a highly accessible and highly conserved site in the HIV genomic RNA and mRNA was inserted within a small RNA element that directs localization of the transcripts into the nucleolus (Figure 3) [23]. Since the HIV-encoded regulatory proteins Tat and Rev have nucleolar localization signals, and both bind to HIV RNA, it was reasoned that these proteins could direct trafficking of HIV RNAs into this organelle. The chimaeric ribozyme transcripts were composed of a strong Pol III promoter element that produced high levels of the transcript, which was localized primarily in the nucleolus.

These nucleolar ribozymes were positioned to bind to and cleave HIV-1 RNAs as they trafficked through the nucleolus, and in fact provided nearly complete inhibition of HIV replication in cell culture [23]. We have recently exploited a different mechanism for specific intracellular trafficking of ribozymes by making fusions of a ribozyme with different elements from the U3 small nucleolar RNA (Figure 4). By retaining or eliminating specific elements from the U3 sequence, our ribozyme transcripts can be localized to the nucleolus, the nucleus, or the cytoplasm and nucleus (D. Castanotto and J. J. Rossi, unpublished work). Other strategies for co-localizing ribozymes and substrates to enhance ribozyme function include the use of viral dimerization/packaging domains on ribozyme and target, nuclear versus cytoplasmic localization, and the use of localized mRNA 3´ untranslated regions on ribozymes and their targets [24]. To achieve maximal ribozyme efficacy, it is important to have some knowledge of the intracellular trafficking of the target RNA. In many instances, the RNAs will not have unique intracellular partitioning, and therefore it becomes necessary to test high levels of ribozyme expression in either the cytoplasm or nucleus to determine which is the best compartment for obtaining optimal ribozyme function.

As the use of ribozymes progresses from cell culture systems into animal models, additional control over ribozyme expression will be required. Ribozyme expression can be restricted to specific organs or cell types through the use of tissue-specific promoters. This has been done successfully in tissue culture by using the tyrosinase promoter, which is exclusively expressed in melanocytes [25]. In another example, transgenic mice that carried a ribozyme gene driven by the insulin promoter that was only expressed in the pancreatic b-cell islets were created [26]. Alternatively, inducible promoters, such as those regulated by tetracycline, have shown utility both in cell culture and in animals, allowing ribozyme expression to be turned on and off at will [22].

Ribozyme delivery

Stable intracellular expression of transcriptionally active ribozymes can be achieved by viral vector-mediated delivery. At present, retroviral vectors are the most commonly used in cell culture, primary cells and transgenic animals [27]. Retroviral vectors have the advantage of stable integration into a dividing host cell genome, and the absence of any viral gene expression reduces the chance of an immune response in animals. In addition, retroviruses can be easily pseudo-typed with a variety of envelope proteins to broaden or restrict host cell tropism, thus adding an additional level of cellular targeting for ribozyme gene delivery. Adenoviral vectors can be produced at high titres and provide very efficient transduction, but they do not integrate into the host genome, and consequently, expression of the transgenes is only transient in actively dividing cells. Other viral delivery systems are actively being pursued, such as the adeno-associated virus, alpha viruses and lentiviruses. Adeno-associated virus is attractive as it is a small, non-pathogenic virus that can stably integrate into the host genome. An alpha virus system, using recombinant Semliki Forest virus, provides high transduction efficiencies of mammalian cells along with cytoplasmic ribozyme expression [28].

Another vehicle for the ex vivo delivery of ribozyme genes is cationic lipids [27]. Since there is a variety of formulations for these lipids, it is usually best to test a panel of lipids to determine which ones provide the highest efficiency of gene transfer with the least toxicity.

Functional genomics and target validation

Ribozymes can be used to inactivate specific gene expression, and therefore can be used to help identify the function of a protein or the role of a gene in a functional biochemical pathway. Target validation is an increasingly important tool in basic biological research as well as in drug development. With the recent completion of the human genome sequencing initiative, there are tens of thousands of transcriptomes that have no assigned function.

Ribozymes provide a facile and highly specific tool for interfering with the expression of these transcripts to monitor their biological function.

Ribozyme-mediated target validation can also be used to identify specific members of a protein family that are involved in a specific phenotype. Carefully designed ribozymes can selectively knock down expression of each protein in a gene family.

Concluding remarks

For ribozymes to become generally useful surrogate genetic tools and realistic therapeutic agents, several obstacles first need to be overcome. These obstacles are the efficient delivery to a high percentage of the cell population, efficient expression of the ribozyme from a vector or intracellular ribozyme concentration, co-localization of the ribozyme with the target, specificity of ribozyme for the desired mRNA, and an enhancement of ribozyme-mediated substrate turnover. As our knowledge of RNA structure (secondary and tertiary) increases, we will be able to target RNAs more rationally, which may help with the problems of specificity. At the same time, the understanding of the physical localization of RNA in cells and its tracking as it moves from the nucleus to cytoplasm will also help in ensuring co-localization of the ribozyme and target.

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Figure legends

Figure 1 Ribozyme-mediated down-regulation of MDM2 results in arrested cell growth. Three ribozymes (Rz 1, Rz 2 and Rz 3) that were targeted to different sequences in MDM2 were expressed from a retroviral vector using a Pol III $tRNA^{Val}$ promoter. The functional ribozymes (W) arrested cell growth, whereas mutant-ribozyme-expressing (m) and control cells continued to proliferate.

Figure 2 Northern blot analysis of ribozyme-mediated down-regulation of the DMPK mRNA from a patient with myotonic dystrophy. The ribozymes were expressed from a nuclear $tRNA^{Met}$ promoter. The wild-type (wt) and mutant ribozymes were transfected into differentiated myoblasts from a patient with myotonic dystrophy who harboured more than 750 CUG repeats in the DMPK mRNA. Both the mutant CUG-repeat-containing transcript (5.1 kb) as well as the non-repeat-containing transcript (2.8 kb) were cleaved, but the cleavage of the mutant transcript resulted in elimination of nuclear foci of DMPK mRNAs (M.-A. Langlois, N. Lee, J. J. Rossi and J. Puymirat, unpublished work). The lanes of the gel are as indicated. Arrows indicate functional ribozyme cleavage products.

Figure 3 Nucleolar anti-HIV-1 ribozymes. The anti-HIV-1 ribozyme was inserted within the apical loop of the small nucleolar RNA (snRNA) U16. The chimaeric U16-ribozyme (U16Rz) was placed behind the Pol III U6 snRNA promoter. Transcripts of the chimaeric RNA localize to the nucleolus, as shown by *in situ* hybridization and co-localization with the small nucleolar RNA U3. *In situ* hybridization was performed in transfected 293 cells. The ribozyme probe was fluorescein-labelled (green), whereas the U3 probe was labelled with Cy3 (red). Reproduced with permission from [23].

Figure 4 Design of U3 small nucleolar RNA–ribozyme chimaeric transcript. The various domains of U3 that specify discrete intracellular partitioning are illustrated. The chimaeric RNAs can be expressed from either Pol II or Pol III promoters, and *in situ* hybridization analyses demonstrate discrete intracellular partitioning dependent upon U3 domains included in transcriptional units (D. Castanotto and J. J. Rossi, unpublished work).

Figures

FIGURE 1

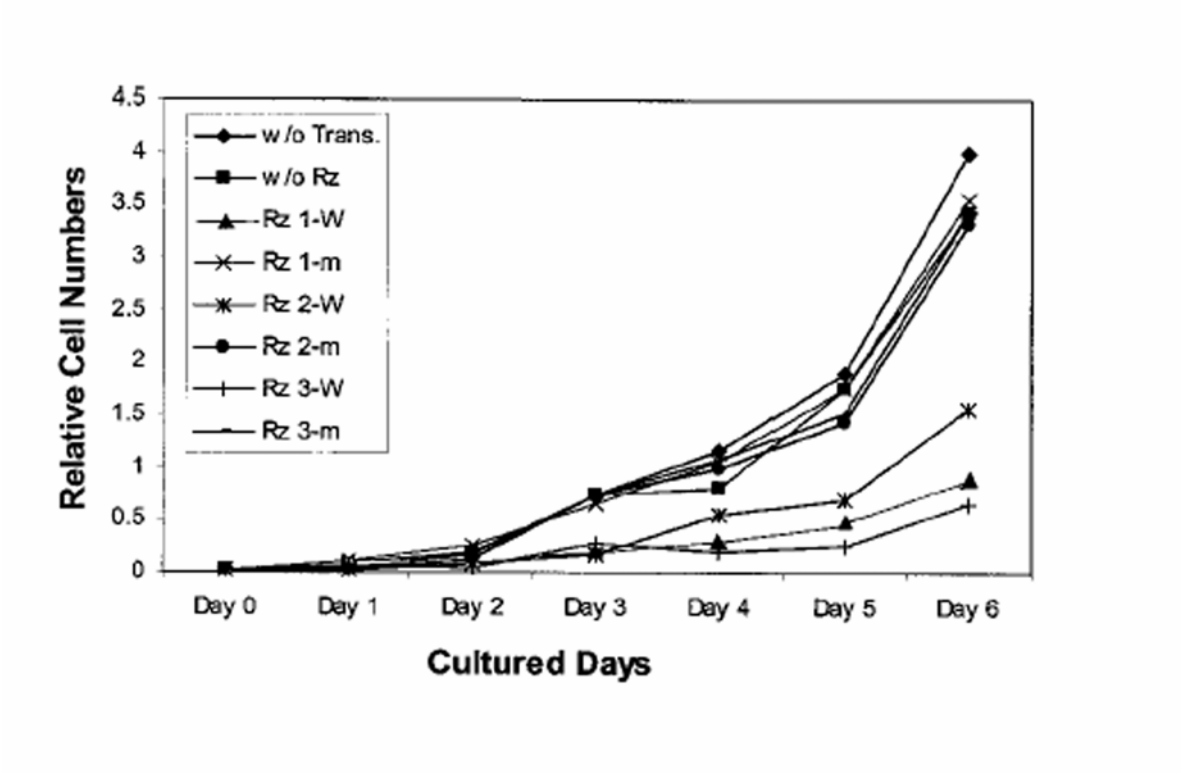


FIGURE 2

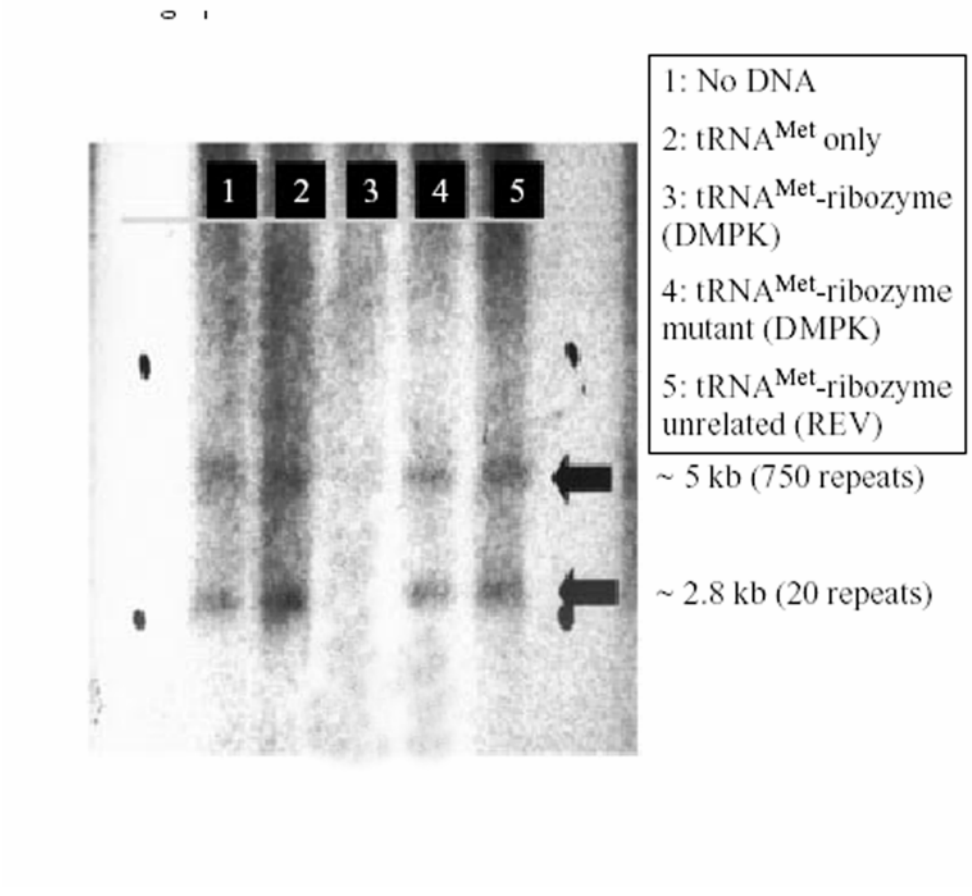

1
2
3
4
5
1: No DNA
2: tRNA^{Met} only
3: tRNA^{Met}-ribozyme (DMPK)
4: tRNA^{Met}-ribozyme mutant (DMPK)
5: tRNA^{Met}-ribozyme unrelated (REV)
~ 5 kb (750 repeats)
~ 2.8 kb (20 repeats)

FIGURE 3

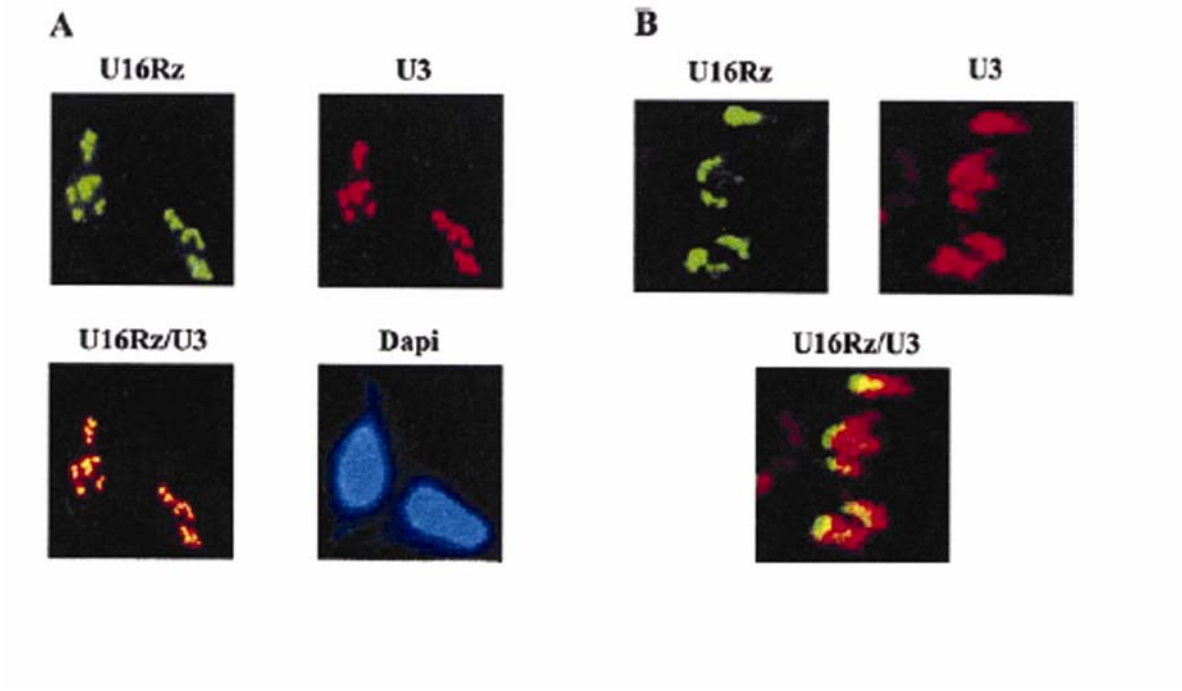
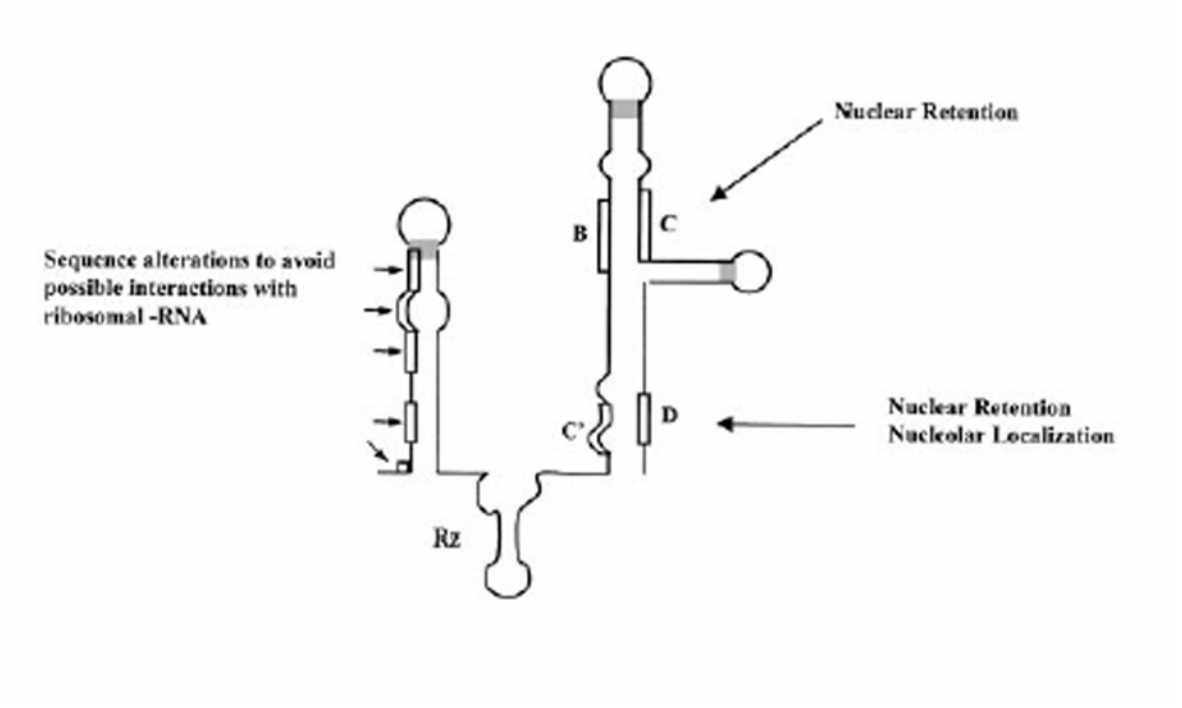


FIGURE 4


Nuclear Retention
Sequence alterations to avoid possible interactions with ribosomal -RNA
B
C
C'
D
Nuclear Retention
Nucleolar Localization
Rz