IDMC-3

THE 3RD INTERNATIONAL MYOTONIC DYSTROPHY CONFERENCE

October 9-11, 2001

The Miyako
Kyoto
Japan

The 3rd International Myotonic Dystrophy Conference

October 9 – 11, 2001
The Miyako, Kyoto, Japan

Scientific Organization Committee

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Association Francais contre les Myopathies (AFM),
The Muscular Dystrophy Association (MDA),
Hunter Research Fund,
The Osaka Medical Research Foundation for Incurable Diseases,
The Aino Hospital Foundation.

Welcome to IDMC-3 Kyoto 2001

It is my great pleasure to welcome you to the 3rd International Myotonic Dystrophy Conference (IDMC-3) in autumn Kyoto. IDMC is a unique international meeting devoted to DM and the participants included both biologists and clinicians.

Originally, with different expertise the IDMC-1 was held in Paris, France in 1997 with the support from the Association Francais contre les Myopathies (AFM) and the Muscular Dystrophy Association (MDA) to stimulate new scientific collaborations and contribute to the discovery of new therapeutic approaches. The IDMC-2 held in Research Triangle, North Carolina, U.S.A. in 1999 was another great success.

In the recent two years, remarkable progress has been achieved in the research on DM and its related issues, which is expected to contribute to the better understanding of the molecular mechanisms underlying the physiopathogenesis of DM and the developments of new therapeutic approaches.

The IDMC-3 promises to give every participant an opportunity to report on new findings, exchange views and experiences, and have fruitful discussions.

We hope that you will enjoy the scientific sessions and the social programs, and your stay in Kyoto is memorable.

Nakaaki Ohsawa, M.D.
Chairman of the Organizing Committee,
IDMC-3, Kyoto 2001

PROGRAM

Tuesday - October 9

A.M.

Welcome ---------- **8:45 - 9:00**

Ohsawa, N., Junien, C., Ashizawa, T.

Invited lectures (1) ---------- **9:00 - 11:00**

I. Applied molecular genetics in myotonic dystrophy
Harper, P.S.

II. Instability of trinucleotide repeats
Oostra, B.A.

III. Signal transduction and physiological function of Rho-associated kinase, ROCK
Narumiya, S.

VI. Lens development and cataractogenesis
Eguchi, G.

Triplet repeat instability / genome organization ---------- **11:00 - 12:20**

Chairpersons: *Gourdon, G., and Monckton, D.*

1. Trans-acting modifiers of expanded triplet repeat stability
Monckton,D.G., Fortune,M.T., Fotheringham,M., Gould,F.K., Gomes-Pereira,M., Ingram, L., *McAbney, J.P.*

2. A bias for DM1 CTG/CAG repeat expansions in a primate DNA replication system: evidence for a *cis*-element
Pearson, C.E., Nichol, K., Wang, Y.-H., Cleary, J.

3. Molecular mechanisms of genetic instabilities of CTG/CAG repeats associated with myotonic dystrophy
Bacolla, A., Bowater, R.P., Gellibolian, R., Iyer, R.R., Jakupciak, J., Jaworski, A., Larson, J.E., Napierala, M., Ohshima, K., Parniewski, P., Pluciennik, A., *Wells, R.D.*

4. Somatic cell-specific instability of an inserted (CTG)84 repeat at the mDMPK locus is prevented by MSH3 deficiency
Nelen, M.R., van den Broek, W.A., Groenen, P.J.T.A., Wansink, D.G., te Riele,H., *Wieringa,B.*

5. Molecular mechanisms of CTG repeat instability in DM1 transgenic mice
Savouret, C., Seznec, H., Brisson, E., Junien, C., Gourdon, G.

6. "Mitotic Drive" of expanded CTG repeats in myotonic dystrophy type 1 (DM1)
Khajavi, M., Tari, A.M., Patel, M.B., Tsuji, K., Siwak, D.R., Meistrich, M.L., Terry, N.H.A., *Ashizawa, T.*

7. CTG repeat instability in human DM1 germ cells
Martorell, L., Monckton, D.G., Baiget, M.

8. Somatic Instability of a (CTG)43 pre-mutation allele in an asymptomatic carrier and two myotonic dystrophy type 1 homozygous patients
Abruzzese, C., *Costanzi Porrini, S.*, Mariani, B., Gould, F.K., McAbney, J.P., Monckton, D.G, Ashizawa, T., Giacanelli, M.

9. A new evidence of somatic instability in myotonic dystrophy type 1 (DM1)
Kinoshita, M., Komori, T., Ohtake, T., Kasahara, N., Nakamura, S., Matsuda, A., Osanai, R., Mitarai, T., Hirose, K.

Lunch time ---------- **12:20 - 13:20**

P.M.

CUG-BP / RNA with expanded repeats ---------- 13:20 - 15:30

Chairpersons: *Cooper, T., and Thornton, C.*

10. Comparison of three methods for the extraction of mutant myotonic dystrophic protein kinase mRNAs (DMPK mRNAs).
    Cossette, L., Doucet, G., *Puymirat, J.*

11. Conditional *in vivo* and *in vitro* models of toxic CUG expansions
    Haworth, C., *Monckton, D.G.*

12. *cis*- and *trans*-effect of the reporter gene containing expanded CTG repeat in neuronal and glial cultured cell lines
    Furuya, H., Kikuchi, H., Yamada, T., Kira, J.

13. Trans-dominant effect of the myotonic dystrophy CTG expansion causes muscle and brain anomalies in mice transgenic for the human DM1 region
    Seznec, H., Agbulut, O., Sergeant, N., Savouret, C., Ghestem, A., Tabti, N., Willer, J.C., Ourth, L., Duros, C., Brisson, E., Butler-Browne, G., Delacourte, A., Junien, C., *Gourdon, G.*

14. Alternative splicing of DMPK is affected in *cis* in DM1: hints for mechanisms for nuclear retention?
    Hamshere, M., Gibb, A.

15. Mis-splicing of Clc-1 mRNA in a transgenic model of myotonic dystrophy
    Thornton, C.A., Mankodi, A., Takahashi, M.P., Beck, C., Cannon, S.C.

16. Characterization of skeletal muscle hyperexcitability in a transgenic mouse model of myotonic dystrophy
    Takahashi, M.P., Mankodi, A., Thornton, C.A., Cannon, S.C.

17. Aberrant regulation of Clc-1 chloride channel alternative splicing in DM1 skeletal muscle: a likely cause of myotonia
    Savkur, R.S., Charlet, B.N., Singh, G., Philips, A.V., Grice, A.E., *Cooper, T.A.*

18. Involvement of muscleblind proteins in the myotonic dystrophies
    Swanson, M.S., Urbinati, C.R., Teng-umnuay, P., Nykamp, K.R., Kanadia, R.N., Non, P.J., Stenberg, M.G.

19. Functional analysis of CUG-BP1 and EXP/MBNL
    Kino, Y., Sasagwa, N., Usuki, F., Ishiura, S.

20. *In vivo* co-localisation of MBNL protein with DMPK expanded-repeat transcripts
    Fardaei, M., Larkin, K., Hamshere, M.G., *Brook, D.J.*

21. Molecular basis for impaired differentiation in myotonic dystrophy
    Timchenko, N.A., Iakova, P., Cai, Z.-J., Smith, J.R., *Timchenko, L.T.*

22. Identification of the CUG repeats binding proteins in HeLa and Myoblast
    Kim, D., Langlois, M-A., Puymirat, J., Rossi, J.J.

Tea break ---------- **15:30 - 15:50**

DMPK ---------- **15:50 - 17:20**

Chairpersons: *Epstein, H., and Wieringa, B.*

23. Characterization of monoclonal antibodies against DMPK and application to changes in DMPK levels during development and in myotonic dystrophy
Lam, L.T., Furling, D., Nguyenthi, M., Butler-Browne, G., *Morris, G.E.*

24. C-terminal and internal VSGGG peptide motifs confer distinct properties to myotonic dystrophy protein kinase (DMPK) isoforms
Wansink,R., van Herpen, R.E.M.A., Coerwinkel, M., van den Broek, W., Groenen, P.J.T.A., Wieringa, B.

25. Expression of DMPK in the autopsied skeletal muscle with severe congenital myotonic dystrophy (cDM)
Tanaka, H.,Shimokawa, M, Mizusawa, H., Arai, H., Yamagata, H., Miki, T., Ishiura, S., *Kobayashi, T.*

26. Ubiquilin-1 binds to myotonic dystrophy protein kinase (DMPK) and modulates its kinase activity and degradation.
Nagamitsu, S., Horvath, G., Barral, J.M., Liu, F., Zhang, R., Epstein, H.F., *Ashizawa, T.*

27. Regulation of serum response factor by myotonic dystrophy protein kinase (DMPK)
Iyer, D., Belaguli, N., Fluck, M., Wei, L., Rowan, B., Weigel, N., Booth, F., Epstein, H., Schwartz, R., *Balasubramanyam, A.*

28. DMPK: Regulator of cytoskeletal membrane dynamics
Epstein, H.F., Liu, F., Zhang, R., Shimizu, M., Hartshorne, D.J.

29. DMPK in for synaptic plasticity of the hippocampus
Epstein, H.F., Liu, F., Wieringa, B., Kasten, M.R., Schulz, P.

30. Abnormal cardiac Na channel gating in mice lacking DMPK
Lee, H.C., Patel, M.K., Reddy, S., Mounsey, J.P., *Moorman, R.*

31. Expression of human DM protein kinase (DMPK) in *Schizosaccharomyces pombe*
Sasagawa, N., Ishiura, S.

Dinner party ---------- **18:30 -**

Wednesday - October 10

A.M.

Invited lectures (2) ---------- **9:00 - 10:00**

V. Molecular mechanisms of insulin resistance
Kadowaki, T

VI. Regulation of muscle cell differentiation, growth and apoptosis
Walsh, K.

Tea break ---------- **10:00 - 10:20**

Six5 / neighbouring genes ---------- **10:20 - 11:20**

Chairpersons: *Johnson, K., and Reddy, S.*

32. A panel of monoclonal antibodies against human Six5 identifies a 100kD nuclear protein in human cell lines and tissues
Pham, Y.C.N., Nguyenthi, M., Hamilton, G.M., Johnson, K.J., Morris, G.E.

33. The *Drosophila* homologue of the myotonic dystrophy associated gene, SIX5, is required for muscle and gonad development
Hamilton, G.M., Kirby, R.J., Finnegan, D.J., Jarman, A.P., *Johnson, K.*

34. The murine Six5 locus is essential for gametogenesis
Sarkar, P.S., Han, J., *Reddy, S.*

35. Involvement of SIX5 in DM pathogenesis
Kawakami, K, Ozaki, H. Tapscott, SJ. Sato S., Ikeda, K.

36. Transcriptional targets of SIX5
Sato, S., Nakamura, M., Bergstrom, D.A., Tapscott, S.J., Tomarev, S., Ibaraki, N., Kawakami, K.

37. Does "painting" of chromosome 19 by (CUG)n-containing DMPK mRNA cause the multisystem phenotype of myotonic dystrophy? A new hypothesis of long-range *cis* autosomal inactivation
Junghans, R.P., Ebralidze, A., Tiwari, B.

DM2 / PROMM ---------- **11:20 - 12:10**
Chairpersons: *Krahe, R., and Ranum, L.*

38. Executive-frontal lobe dysfunction in proximal myotonic myopathy (PROMM): Evidence for dopamine modulation of limbic-prefrontal cortex circuits?
Meola, G., Sansone, V., Cotelli, M., Cattaneo, E., Cappa, S., Scarone, S., Dragoni, C., Perani, D.

39. Microarray-based expression profiling of gene expression changes in DM1 and DM2
Krahe, R., Colella, S., Ashizawa, T., Udd, B., Wright, F.A., Virtaneva, K.

40. Proximal myotonic dystrophy (PDM) and progressive myotonic myopathy (PROMM) in Finnish and Italian kindreds: linkage to and linkage disequilibrium at the DM2 locus in 3q21.3 in two separate founder populations
Krahe, R., Holloway, M., McWhinney, S., Meola, G., Sansone, V., Udd, B.

41. Molecular genetics of DM2 clarify pathogenic mechanism(s) of myotonic dystrophy
Ranum, L.P.W., Liquori, C.L., Moseley, M.L., Jacobsen, J.F., Kress, W., Naylor, S., Ricker, K., Day, J.W.

42. Clinical and genetic correlations of myotonic dystrophy type 2.
Day, J.W., Ricker, K., Dick, K., Rasmussen, L., Dalton, J., Jacobsen, J.F., Liquori, C.L., Kress, W., Schneider, C, Phillips, A., Savkur, R., Cooper, T.A., Moseley, M.L., Ranum, L.P.W.

Lunch time ---------- **12:10 -** ***Free time for sightseeing***

Evening session

DM phenotype ---------- **19:00 - 20:40**
Chairpersons: *Butler-Browne, G. and Miki, T.*

43. Myotonic dystrophy: a simple mendelian disorder as model of hypercomplexity
Brisson, D., Houde G., Vohl, M.C., St-Pierre, J., Mathieu, J., *Gaudet, D.*

44. Large CTG expansion altered the proliferative capacity of DM-1 satellite cells
Furling, D., Mouly, V., Barbet, J.P., Butler-Browne, G.S

45. Congenital myotonic dystrophy : defective satellite cells
Butler-Browne, G.S., Furling, D., Coiffier, L., Barbet, J.P., Lacau St Guily, J., Taneja, K., Gourdon, G., Junien, C., Mouly, V.

46. Increase activation of subcortical structures in myotonic dystrophy: a functional MRI study
Caramia, F., Mainero, C., Gragnani, F., Bucci, E., Ceschin, V., Morino, S., Iannetti, G., Pantano, P., Bozzao, L., *Antonini, G.*

47. Dysregulation of human brain microtubule-associated tau mRNA maturation in myotonic dystrophy type 1
Sergeant, N., Schraen-Maschke,S., Ghestem,A., Wattez,A., Maurage,C.A., Seznec,H.,Gourdon,G., Sablonniere, B., Delacourte, A.

48. Abnormal cardiac magnetic resonance imaging findings in myotonic dystrophy with inducible ventricular tachyarrhythmias
Duboc, D., Lazarus, A., Eymard, B., Carlier, P., Varin, J., Becane, H.M., Laforet, P., Legmann P., Vignaux, O.

49. Correlation between cardiac conduction disease and CTG expansion size
Hilton-Jones, D., Nixon, J., Clark, N., Kelion, A., Forfar, C.

50. Contractile activity of cardiac myocytes isolated from Dmpk KO mice
Pall, G.S., Johnson, K.J., Smith, G.L.

51. Genotype-phenotype correlation of otolaryngologic findings in myotonic dystrophy type 1
Osanai, R., Kinoshita, M., Hirose, K.

52. High resolution haplotype analysis in the DMWD-DM1-SIX5 region
Yamagata, H., Miki, T.

Thursday - October 11

A.M.

Diagnosis of DM1/DM2 ---------- 9:00 - 9:30

Chairpersons: *Day, J., and Udd, B.*

53. Myotonic dystrophy: Frequency and characteristics of diagnosis missing by physicians in France
Eymard, B., Dobon, I., Bassez, G., Laforet, P.

54. Outcomes of presymptomatic testing for myotonic dystrophy
Harper, P.S., Fokstuen, S.

55. Type 2 fiber atrophy in DM2-linked PROMM - diagnostic distinction to myotonic dystrophy (DM1)
Udd, B., Vihola, A., Haapasalo, H., Paetau, A., Krahe, R.

Clinical rating scale / evaluation ---------- 9:30 - 9:50

Chairpersons: *Hilton-Jones, H., Mathieu, J. and Thornton, C.*

56. Prevalence of excessive daytime sleepiness in myotonic dystrophy
Orlikowski, D., Laforet, P., Quera Salva, T., Duboc, D., Lofaso, F., Gajdos, P., Raphael, J.C., Eymard, B., *Annane, D.*

57. The electromyographic study of dehydroepiandrosterone sulfate in patients with myotonic dystrophy
Ito, T., Sugino, M., Ishida, S., Tanaka, T., Hanafusa, T., Ohsawa, N.

Tea break ---------- **10:00 - 10:20**

Therapeutic approach / Treatment ---------- **10:10 - 11:40**
Chairpersons: *Hilton-Jones, H., Mathieu, J., and Thornton, C.*

58. The effect of dehydroepiandrosterone sulfate on myotonia: Intracellular study
Kurihara, T., Kishi, M., Nemoto, H.

Comments : *Imoto, K.*

59. Molecular mechanisms of dehydroepiandrosterone-sulfate effects on skeletal muscle cells
Furutama, D., Fujita, A., Tanaka, T., Tsuji, K., Hanafusa, T., Ohsawa, N.

60. Developing a tissue culture model of CTG instability using DM1 patient cells
Pearson, C.E., Lau, R., Chitayat, D., Yang, Z.

61. Chemotherapeutically induced deletion of expanded triplet repeats
Sinden, R.R., Hashem, V.I., Tsuji, K., Khajavi, M., Ashizawa, T.

62. The use of RNA antisense as a potential gene therapy for myotonic dystrophy (DM)
Puymirat, J., Doucet, G., Cossette, L., Timchenko, L.

63. A novel RNA targeting approach for the repair of the myotonic dystrophy defect
Yamasaki, E.N., Everatt, L., Wood, M.J.A., *Phylactou, L.A.*

64. Excessive daytime sleepiness in myotonic dystrophy
Hilton-Jones, D., Talbot, K., Stradling, J.

65. A conceptual framework to implement a comprehensive systemic health care program in myotonic dystrophy
Mathieu, J., Gaudet, D., Gourdon, G., Noreau, L.

Highlight and summary ---------- **11:40 - 12:10**
Chairpersons: *Ashizawa, T. and Wieringa, B.*

Disucussion for all topics ---------- **12:10 - 12:30**
Chairpersons: *Ashizawa, T. and Wieringa, B.*

Sayonara Kyoto
Ohsawa, N.

I. Applied molecular genetics in myotonic dystrophy

Harper PS

University of Wales College of Medicine, Cardiff, United Kingdom

Recognition of the expanded CTG of myotonic dystrophy has had major practical applications in genetic counselling and testing, but important areas remain uncertain. The following areas are among those needing full discussion:

1. How close and how useful is the correlation between phenotype and repeat number?
2. How accurate is molecular analysis in prenatal and pre-implantation diagnosis?
3. Should molecular testing be undertaken on healthy children at risk?
4. What preparation and counselling is needed for pre-symptomatic testing?
5. Is there a case for population screening for myotonic dystrophy?

As much debate as possible is needed on these and related topics before clear practice guidelines can be given.

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II. Instability of trinucleotide repeats

Oostra BA

Dept Clinical Genetics, Erasmus University, Rotterdam

The fragile X syndrome was the first identified disease of a growing list of disorders known to be caused by the so-called "dynamic mutations", resulting from the instability and expansion of trinucleotide repeats.
In fragile X syndrome large instability to the disease size (full mutation) can only occur via maternal transmission. Analysis of intact ovaries from full mutation female fetuses showed, that only full mutation alleles can be detected in oocytes but in the unmethylated state, in contrast to the methylated state of somatic tissue. On the other hand sperm of affected males only show premutations , while the testes of a 13 week full mutation fetus show the presence of a full mutation. This suggest that the presence of premutation sperm in affected males is due to full mutation contraction to the premutation in the immature testis with subsequent selection for these spermatogonia.
To study this event in more detail we have generated a mouse model with a long CGG repeat. Instability of the repeat is noticed although at a lower level than is seen in humans. Surprisingly, paternal transmission showed a higher instabilty than in female transmissions. The transgene was introduced into backgrounds of mice that were knockout for a number of repair genes. Surprisingly, the repeat seems to be more stable in such a mouse system.
Instability of the CGG repeat in fragile X syndrome will be compared to the instability seen of the CAG or CTG repats in other trinucleotide repeat disorders.

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III. Signal transduction and physiological function of Rho-associated kinase, ROCK

Narumiya S

Department of Pharmacology, Kyoto University Faculty of Medicine, Sakyo-ku, Kyoto 606-8501, Japan

The ROCK family of Rho-associated protein kinases is a group of the effectors of the small GTPase Rho, and consists of ROCK-I (ROKβ) and ROCK-II (ROKα, Rho-kinase). ROCKs contain a serine/threonine kinase domain in its N-terminus, followed by a coiled-coil structure about 600 amino acid long, and a cysteine-rich zinc finger and a PH domain in the C-terminus. The N-terminal region containing a kinase domain and a part of coiled-coil structure shows strong homology to myotonic dystrophy kinase over 500 residues. Then, what is the function of ROCK in the cell? Rho works as a switch in various cellular processes such as stimulus-induced cell to substrate adhesion, contraction, cytokinesis and nuclear signaling. Rho is also involved in cell growth and transformation. These Rho actions are performed in part through the reorganization of the actin cytoskeleton. For example, the cell-substrate adhesion is exerted through the formation of focal adhesions and actin stress fibers in cultured fibroblasts. The involvement of ROCK in these Rho-mediated processes has been examined and their signal transduction has been analyzed by expressing ROCK mutants and by using a specific ROCK inhibitor, Y-27632. Y-2763 is a synthetic pyridine derivative, and competes with ATP in binding to ROCK. However, Y-27632 shows specific inhibition of ROCK at lower μM concentrations in cells and tissues, indicating that its inhibition mechanism is not a simple competition with ATP. These analyses have identified that ROCK enhances the actomyosin contractility through phosphorylation and downregulation of myosin phosphatase, and also, possibly, by direct phosphorylation of myosin light chain. ROCK also phosphorylates LIM-kinase and type I Na-H exchanger; the former leads to phosphorylation of cofilin, resulting in inhibition of actin disassembly, and the latter may result in F-actin anchoring to cell membrane. These ROCK actions combined together induce actomyosin bundling and contraction, and contribute to formation of focal adhesions and stress fibers, growth cone collapse and neurite retraction, and smooth muscle contraction. ROCK has also been reported to phosphorylates occludin and regulate tight junction. All of these actions are elicited by ROCK activated downstream of Rho. However, ROCK has recently been shown to be activated also by proteolytic cleavage with a caspase and contribute to formation of membrane blebs in apoptotic cells. Then, what is the physiological and pathological significance of ROCK-mediated cell responses in the body? Y-27632 has been again very useful to uncover it. This compound attenuates pathological processes involving smooth muscle contraction or cell motility. The former examples are Y-27632-induced attenuation of hypertension in various rat models, and inhibition of contractile response of sensitized bronchial smooth muscles. Y-27632 also affects the pathological processes involving cell adhesion and migration such as tumor metastasis and invasion, vascular remodeling and lung fibrosis. The experiment with Y-27632 also indicates the involvement of ROCK in Ras-induced malignant cell transformation.

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IV. Lens development and cataractogenesis

Eguchi G

Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan

The lens of the vertebrate develops from the head surface ectoderm of the early embryo through inductive effects of factors released by the eye rudiment, optic vesicle which organizes main part of the eye. This organ, consisting of a monolayered epithelium designated as lens epithelium and lens fibers which differentiate successively from lens epithelial cells, is totally lacking in the circulatory system. The physiological conditions of lens should be maintained by cell surface functions such as active transport of lens epithelial cells. In addition, differentiation from lens epithelial cells to lens fibers continues throughout life. Therefore, the physiological state of lens must be deeply dependent upon its environmental conditions. Actually, compositional changes of the viterous humor, major components of which originate in the blood plasma, readily induce cataractogenesis as observed in diabetic patients. Causal mechanisms and genetic background are highly complicated so that search and research of genes responsible for cataractogenesis must be highly important for crucial understanding of cataractogenesis. In this occasion, I will introduce some representative case of cataractogenesis referring to characteristic development and differentiation of the lens.

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1. *Trans*-acting modifiers of expanded triplet repeat stability

Monckton DG, Fortune MT, Fotheringham M, Gould FK, Gomes-Pereira M, Ingram L McAbney JP

Institute of Biomedical and Life Sciences, Division of Molecular Genetics, Anderson College, University of Glasgow, 56 Dumbarton Road, Glasgow G11 6NU, U.K.

In myotonic dystrophy type 1 expansion of the CTG repeat in the germline leads to intergenerational increases in repeat length and the resultant clinical anticipation. Similarly, age dependent, tissue specific, expansion biased somatic mosaicism contributes toward the tissue specificity and progressive nature of the symptoms. The sex dependent nature of germline mosaicism and the specificity of somatic instability must be mediated by tissue specific trans-acting genetic modifiers. Previously, we have also revealed differences in repeat length variability between individuals which cannot be accounted for by age or allele length, implying the existence of additional individual specific trans-acting modifiers. In attempts to further define the role of, and identify the nature of, trans-acting modifiers of repeat stability, we are using a combination of approaches including the analysis of expanded repeat length variation in: DM1 patients; patients with other inherited expansion disorders; normal humans with expansions at the non-pathogenic CTG18.1 and ERDA1 loci; normal tissue and tumours of patients with hereditary non-polyposis colon cancer; transgenic mouse models carrying large expanded repeats; mouse models of DNA repair deficiencies; and mouse tissue culture models. These analyses have revealed additional evidence for a crucial role of trans-acting modifiers in humans, specifically implicating mutant alleles in the mismatch repair pathway. Similarly, use of mouse models have indicated that the Pms2 mismatch repair gene is required for maintaining a high rate of somatic expansion and for preventing large deletions. Whilst detailed analysis of a tissue culture model has established that tissue specific differences cannot be accounted for by cell division rates. Overall, these data suggest that repeat expansion may be mediated by multiple rounds of inappropriate DNA repair in non-diving cells.

WIP

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2. A bias for DM1 CTG/CAG repeat expansions in a primate DNA replication system: evidence for a *cis*-element.

Pearson CE[1)2)], Nichol K[1)], WangY-H[3)], Cleary JD[1)2)]

1) The Hospital for Sick Children, Toronto, ON, Canada
2) University of Toronto
3) Robert Wood Johnson Medical School, Piscataway, NJ, USA.

Expansions of CTG/CAG repeats is the cause of at least 11 human diseases, including myotonic dystrophy, spinalbulbar muscular atrophy and Huntingtons disease. Repeat instability is intimately connected to the length of the repeat tract: Generally (CTG)n tracts from 4 to 25 repeats are stable and tracts from 34-90 repeats are unstable, displaying a bias for expansions. The mechanism of repeat instability is unknown but may involve DNA replication slippage. In addition, cis-elements in the vicinity of the repeat contribute to instability, however the exact nature of these elements remains unknown. One possible cis-element is the location of replication origins relative to the repeat.

We investigated the effect of the location of replication initiation on CTG/CAG stability in a primate DNA replication system. Replication templates were constructed with the SV40 viral replication origin in one of seven locations relative to repeat tracts of non-affected (17 repeats) or premutation (79 repeats) lengths. Templates were transfected and replicated in primate cells (COS1). Following DNA replication, repeat instability was only observed in premutation length templates. Depending on the distance between the SV40 origin of replication and the repeat tract, these templates yielded predominantly expansions or predominantly deletions or remained intact. The relative location of replication initiation was a more critical determinant of repeat instability than was replication direction. All templates with 17 repeats were stable. This is the first system to recapitulate the repeat length effect and the bias for expansions observed in affected families. Furthermore, our results provide the first evidence that may explain the variable levels of CTG/CAG instability observed in different chromosomal contexts.

WIP

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3. Molecular mechanisms of genetic instabilities of CTG•CAG repeats associated with myotonic dystrophy

Bacolla A, Bowater RP, Gellibolian R, Iyer RR, Jakupciak J, Jaworski A, Larson JE, Napierala M, Ohshima K, Parniewski P, Pluciennik A, Wells RD

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The nucleic acid molecular mechanisms responsible for genetic instabilities of CTG•CAG repeat sequences involved in the etiology of myotonic dystrophy are investigated. Expansions and deletions in this triplet repeat sequence (TRS) are mediated by DNA replication and tandem duplication, repair, recombination-gene conversion, or a composite of these mechanisms acting in concert. The slippage of the complementary strands of the repeat sequences to form hairpin loop structures, or slipped conformations, with differing relative stabilities are important components in the mechanism. The inherent conformational properties of these sequences, such as their high degree of flexibility, writhing, and stability of hairpin formation, facilitate the strand slippage. The unusual DNA conformations cause DNA polymerase stalling and hairpin formation, exacerbating the disease-causing mutagenesis. Several genetic/biochemical factors are involved including methyl-directed mismatch repair, nucleotide excision repair, single-strand DNA binding protein, transcription, and DNA polymerase III proofreading.

The recent emphasis of our laboratory stems from the discovery that genetic recombination is a robust mechanism for expanding CTG•CAG repeats. Gene conversion (recombinational repair) is, by far, the most powerful expansion mechanism compared to replication, repair, and tandem duplications. Thus, gene conversion may be the expansion mechanism for myotonic dystrophy.

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4. Somatic cell-specific instability of an inserted $(CTG)_{84}$ repeat at the mDMPK locus is prevented by MSH3 deficiency

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The mechanism of $(CTG)_n$ repeat expansion in myotonic dystrophy patients and its dominant-negative gain-of-function or dosage effect(s) on gene expression is still unknown. We have developed novel DM mouse models containing a "humanized" DMPK allele with either a $(CTG)_{84}$ or normal $(CTG)_{11}$ repeat. Behaviour of these repeats was studied in different tissues over successive generations of these animals. As expected, the $(CTG)_{11}$ repeat was completely stable, both at the somatic and intergenerational level. The $(CTG)_{84}$ repeat however, did show a marginal increase in size during intergenerational segregation and has now expanded to $(CTG)_{105}$ during breeding over 8 successive generations. Moreover, the $(CTG)_{84}$ repeat length shows a conspicuous somatic mosaicism. Whereas in brain, heart, tongue, calf muscle, semen, eyes, ovaries, and liver only minor size changes were observed, we consistently saw much more pronounced repeat expansion (up to $(CTG)_{150}$) in stomach, small intestine, and kidney. Repeat expansion in stomach and the small intestine was predominantly seen in the mucosa cell fraction. This somatic instability is progressive and age dependent.

Length alterations in simple repeats are frequently correlated with defects in individual components of the DNA mismatch repair (MMR) machinery. To study the effect(s) of different types of MMR-activity on repeat behaviour we crossed the $(CTG)_{84}$ allele into a MSH3 -/- or MSH6 -/- deficient background. At six months of age, $(CTG)_{84}$ repeat instability was very obvious in stomach and kidney of MSH6-/- mice. By contrast, somatic length expansion seemed completely blocked in MSH3 deficient animals. Our data are in concordance with the MSH2-mediated DNA-repair effects reported for mice that bear a HD $(CAG)_n$ repeat. Models explaining the distinct effects that MSH2/MSH3 or MSH2/MSH6 complex function has on somatic instability of $(CAG)_n/(CTG)_n$ repeats will be discussed.

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5. Molecular mechanisms of CTG repeat instability in DM1 transgenic mice

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The molecular basis of Myotonic Dystrophy (DM1) is a CTG expansion located in the 3' untranslated region of the DM protein kinase gene (DMPK), characterized by very large intergenerational increases and high levels of somatic instability whose mechanisms are not fully understood. We studied the CTG repeat instability mechanisms in our transgenic mice models carrying 45 kb of the human genomic DM1 region with the three DMWD, DMPK and SIX5 genes and either 20, 55 or 320 CTG. A striking similarity with DM1 was observed in the three models, with a bias towards expansions throughout generations, sex- and size-dependence, and a threshold between 20 and 55 CTG. Moreover, somatic instability is perfectly reproduced, with also a bias towards expansions and no correlation of the level of somatic mosaicism neither with the tissue division rate nor with the level of transcription of the surrounding genes.
Different studies performed in bacteria, yeast and human have demonstrated an influence of DNA repair mechanisms on CTG repeat instability. To investigate this hypothesis, we have crossed our transgenic mice containing 320 CTG with mice knockout for genes belonging to different repair mechanisms, including mismatch repair (Msh2), double-strand break repair (Rad52, Rad54) and non-homologous end-joining (DNA-PKcs). Intergenerational and somatic instability were then assayed over many generations and in numerous tissues at different ages. The most striking result was obtained with the Msh2 gene, as Msh2 -/- transgenic mice showed no more expansion at intergenerational and somatic levels. In contrast, a strong bias towards contraction was observed, showing that Msh2 is necessary for expansions to occur. Moreover, the analysis of the expansion and contraction transmissions, with regards to the parent and kindred Msh2 genotype, suggest that an event of expansion/contraction may take place just after fecundation.
In addition, in order to determine when during spermatogenesis does instability take place, we are studying in our transgenic mice the CTG repeat instability in germ cells from different stages. According to preliminary results, no difference of instability appears between the different germ cell types and sperm. This first results showed that instability therefore occurs in spermatogonia.. Small-pool PCR is in progress to confirm this data.

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6. "Mitotic Drive" of expanded CTG repeats in myotonic dystrophy Type 1 (DM1)

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In myotonic dystrophy type 1 (DM1), an expanded CTG repeat is unstable in somatic and germ line tissues with a strong predilection to expand further. To investigate the mechanism of this expansion bias, 29 DM1 and six normal lymphoblastoid cell lines were single-cell cloned from blood cells of 18 DM1 patients and six normal subjects by limiting dilution. In all 29 cell lines, the expanded CTG repeat alleles gradually shifted toward further expansion by "step-wise" mutations over several passages. Eight of these 29 cell lines yielded a rapidly proliferating mutant with a gain of large repeat size that became the major allele population, eventually replacing the progenitor allele population. By mixing subcloned cell lines with different repeat expansions, we found that cells with larger CTG repeat expansion had a growth advantage over those with smaller expansions in culture. This growth advantage was attributable to increased cell proliferation mediated by Erk1,2 activation, which is negatively regulated by $p21^{WAF1}$. The level of $p21^{WAF1}$ appears to be positively regulated by WT1 in these lymphoblastoid cell lines. This phenomenon, which we designated "mitotic drive," is a novel mechanism that can explain the expansion bias of DM1 CTG repeat instability at the tissue level, on a basis independent of the DNA-based expansion models. The life spans of the DM1 lymphoblastoid cell lines were significantly shorter than normal cell lines. Thus, we propose a hypothesis that DM1 lymphoblastoid cell lines drive themselves to extinction through a process related to increased proliferation. We are currently investigating the mechanism of the shortened life span.

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7. CTG repeat instability in human DM1 germ cells

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Myotonic dystrophy (DM1) is caused by the expansion of an unstable CTG repeat located in the 3'-untranslated region of the *DM protein kinase* gene (*DMPK*). The number of CTG repeats is polymorphic in the general population with a range of 5 to approximately 37 repeats. DM1 patients have expansions of greater than 50 repeats and up to many thousands. The size of the repeat is positively correlated with the severity of the disease and inversely correlated with the age of onset of symptoms.
Dramatic instability with very large intergenerational increases and contractions is observed in DM1 patients.
Detailed studies of somatic mosaicism have revealed that it is tissue specific, biased toward further expansion and continuous throughout the life of an individual.
The trinucleotide repeats instability mechanisms involved in DM1 and in other genetic diseases are unknown.
In order to gain a better understanding of the dynamics of repeat instability in the male germline, we have used sensitive small pool-PCR analyses (SP-PCR) to compare blood and sperm DNA from 21 males, with different age, CTG repeat expansion and clinical form.
Analysis of sperm DNA from control individuals showed that small normal alleles were stable. Sperm samples of DM1 patients revealed both different levels of mosaicism and patterns of distribution for the expanded allele, designing a characteristic pattern in each clinical group. The comparison of these results with those obtained from peripheral blood will allow us to define, in accordance with age and clinical form, specific patterns of mosaicism in somatic and germline cells for DM1 individuals.

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8. Somatic instability of a (CTG)43 pre-mutation allele in an asymptomatic carrier and two myotonic dystrophy type 1 homozygous patients

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Myotonic dystrophy type 1 (DM1) is associated with the expansion of an unstable CTG repeat located in the 3'-untranslated region of the DM protein kinase gene (DMPK). The size of the expanded repeat generally increases in length during parental transmission accounting for the clinical anticipation characteristic of repeat associated diseases. In the general population alleles range from 5 to 37 repeats. The DM1 mutation can be carried by asymptomatic or minimally affected individuals carrying small protomutations, ranging from 50 to 80 repeats. Protomutations are relatively stable during maternal transmission, but passage through the male germline tends to result in further expansion into the full mutation disease range. Pre-mutation alleles, ranging from 38 to 50 repeats, are quite rare in the population and represent the pool from which new DM1 protomutation or full mutations arise. Expanded CTG repeats are unstable in both somatic and germ line tissues with a general bias toward further additions. We here analyse the stability of a maternally inherited (CTG)43 pre-mutation allele in the presence of a full-mutation allele derived from a paternal (CTG)52 proto-mutation allele resulting in (CTG)43/500 and (CTG)43/180 in two affected sons. Small-pool PCR was performed at the single molecule level to measure length heterogeneity of the (CTG)43 pre-mutation allele in peripheral blood DNA and muscle DNA after biopsy in the affected sibs compared to the maternal allele. The (CTG)43 allele was very stable in the mother, with the maximum additions of two repeats and deletions of one repeat. Conversely, in the younger affected sibs the (CTG)43 allele shows a high degree of instability (~70% mutation frequency) resulting in deletions reverting to the normal range (down to (CTG)33) and additions up to the proto-mutated range (up to (CTG)64). The decreased stability of the (CTG)43 allele in the offspring could be due to some influence of the full-mutation allele, possibly revealing some interallelic interaction between expanded alleles.

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9. A new evidence of somatic instability in myotonic dystrophy type 1 (DM1)

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Background: Myotonic dystrophy type 1 (DM1) is an autosomal dominant disorder characterized by a wide variety of clinical features. The mutation responsible for DM1 is an unstable expansion of a CTG triplet repeat ((CTG)n) in the 3' untranslated region of a gene encoding DM protein kinase (DMPK). The somatic instability of (CTG)n expansions has been reported. We therefore investigate (CTG)n in various types of tissue obtained at autopsy from five DM1 patients.
Object and Methods: The (CTG)n sizes in leukocytes in four patients (Case 1, 2, 3, and 4) with typical features were 1000, 1400, 1400, and 2060 repeats (expanded allele sizes 3.0, 4.2, 4.2, and 6.2 kb), whereas that size in one patient (Case 5) without almost clinical signs was about 60-70 repeat (0.1 kb). Samples of 20-28 types of tissue were taken from these patients at autopsy 1.5 to 3 hours postmortem. Southern blot analysis was performed using the method described previously (*Muscle Nerve 19:240, 1996*). Briefly, the genomic DNAs (5 μg) extracted from those tissues and leukocytes were digested with one of two restriction enzymes, Eco R I or Bgl I, then hybridized with a ^{32}P-labeled cDNA 25 probe. In case 5, DNAs extracted from the lymphocytes and various types of tissue were amplified by PCR with primers 101 and 102, then analyzed in a DNA sequencing gel to accurately determine the repeat length of the allele.
Results: In Case 1, 2, 3, and 4, lymphoid tissues including lymph node, spleen, and thymus, had approximately the same (CTG)n sizes as leukocytes. The (CTG)n sizes in the other tissues except cerebellum were uniformly longer than in leukocytes, whereas that size in cerebellum was shorter than in leukocytes. In Case 5, all tissues including cerebellum had approximately the same (CTG)n sizes as leukocytes.
Conclusion: Somatic instability in DM1 patients with a small (CTG) n expansion might be less dominant than that in patients with larger expansions.

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10. Comparison of three methods for the extraction of mutant myotonic dystrophic protein kinase mRNAs (DMPK mRNAs)

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It is now well establish that the mutant DMPK transcripts are sequestrated into the nuclei of DM muscle cells. It has been recently shown that it was essential to purify RNAs through CsCl, rather than with acid guanidium thiocyanate/phenol/chloroform to effectively isolate mutant DMPK transcripts (*Davis et al. Proc Natl Acad Sci, 1997,94,7388-7393*). To determine the best way to isolate mutant DMPK transcripts, we compared three different methods: 1) RNA extraction with TRIzol; 2) RNA extraction with guanidium thiocyanate through CsCL; 3) Poly(A+) extraction after Proteinase K digestion. RNAs and poly(A+) were analyzed by Northern blot using a DMPK cDNA probe. The cellular localization of DMPK transcripts was analyzed by FISH using a Cys3 labeled PNA(CUG)5 probe. Northern analysis revealed that RNA preparation with TRIzol leads to the extraction of about 30 to 40 % of the mutated DMPK mRNAs. Curiously, similar levels of mutants DMPK mRNAs were obtained with guanidiumthiocynate/CsCL. In contrast, the use of proteinase K followed by poly(A+) purification with oligo(dt) leads to a complete extraction of the mutants DMPK transcripts (ratio mutant/normal DMPK mRNA = 1) in differentiated DM myoblasts. By FISH, we did not detected normal DMPK mRNAs in the nuclei of normal myoblasts whereas they were easily detected in the cytoplasm. These results together do not support previous published data showing that 40% of normal DMPK mRNAs are detected in the nuclei and that it was essential to purify mutant DMPK transcripts with Guanidium Thiocynate/CsCL. Finally, our data deomstrate that the best way to prepare DMPK RNA is that involving a digestion with proteinase K followed by a purification of poly(A+).

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11. Conditional *in vivo* and *in vitro* models of toxic CUG expansions

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There are two main hypotheses relating to the pathogenesis of myotonic dystrophy type 1 (DM1), loss of function and gain of function. Alteration in local *DMPK* chromatin structure and nuclear retention of mutant *DMPK* transcripts could lead to haploinsufficiency of DMPK and/or neighbouring genes. Mutant *DMPK* RNA could have a dominant effect by disrupting the delicate balance of RNA binding proteins leading to aberrant processing of RNA transcripts unrelated to *DMPK*. DM1 is a multisystemic disorder and to date it has been difficult to attribute individual aspects of the phenotype directly to a particular mechanism, either loss or gain. Therefore it is still unclear whether a number of complex mechanisms operate in concert to bring about the considerable clinical variability or whether all symptoms could result from a single cause. In order to delineate cause and effect of an expanded CUG RNA in DM1, we are producing a conditional dominant CUG repeat expressing mouse model using the cre-lox system. Cre, a site-specific recombinase isolated from bacteriophage P1, recognises a 34bp DNA sequence termed loxP. DNA situated between two loxP sites is excised by cre therefore expression of an expanded CUG RNA can be controlled by the removal of a transcription stop signal situated between two loxP sites. This system will enable us to activate the expression of an expanded CUG RNA in a tissue or temporally specific manner by genetic crossing with cre-expressing mice. To avoid positional effects between the control and the expanded repeat transgene we are using embryonic stem (ES) cell technology to target to identical sites within the genome. In addition to transgenic mouse production, we are able use the resulting ES cell-lines as a conditional expressing *in vitro* model. By taking advantage of the pleuripotency of these cell-lines it is possible to study the effects of expanded repeat expression before, during and after differentiation into DM1 affected lineages which could shed some light on the mechanism involved in congenital DM1 pathogenesis. So far, in a tissue culture model, we have shown that the expanded $(CUG)_{250}$ transgene results in the formation of nuclear foci upon cre activation whereas the control $(CUG)_5$ transgene does not. We are presently studying the effect of $(CUG)_{250}$ expression on RNA processing *in vitro*.

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12. *cis*- and *trans*-effect of the reporter gene containing expanded CTG repeat in neuronal and glial cultured cell lines

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Objective: To study the effect of the myotonic dystrophy (DM) CTG expansion in the 3'-untranslated region (3'-UTR) of the DM protein kinase gene (DMPK) *in vitro*, we have established a cell culture model system and examined its effect to cytoskeletal proteins in neuronal and glial cell lines to explore the CNS disturbance in DM.
Materials and Methods: We injected luciferase reporter gene constructs containing DM patient-derived CTG or CAG-repeat (135 repeats) in its 3'-UTR into PC12 (the rat neuronal cell line) and U373 (human glioma cell), then, assayed its luciferase activity at appropriate time points. After transfecting these plasmid constructs, cells grown on glass coverslips were analyzed using antibodies for cytoskeletal proteins containing tau-protein using immunocytochemical methods.
Results: Our data shows that the CTG-repeat expanded construct, not CAG-repeat, have a negative *cis*- effect on reporter gene expression in both type of cells two days after infection. Morphological and immunohistochemical analysis show abnormal cytoskeletal structures and low signal staining of tau-protein in both PC12 and U373 cell line after transfection of repeat-containing plasmid constructs especially in CTG repeat.
Discussion: These results are consistent with the finding of low expression of DMPK in muscular tissue of DM patients, suggesting that low expression of expanded CTG repeat-containing genes is a general phenomenon not only in myoblast cell but in neuronal cell lines. In addition to this cis-acting effect, *trans*-acting effect is also observed even in neuronal cell. We assumed that the transcript of expanded CTG-containing gene acts not only in cis- to reduce protein production but also in *trans* to inhibit neurogenesis or to facilitate neuro-degeneration in CNS as well as myogenesis in muscular tissue.

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13. Trans-dominant effect of the myotonic dystrophy CTG expansion causes muscle and brain anomalies in mice transgenic for the human DM1 region

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The autosomal dominant mutation causing Myotonic Dystrophy (DM1) is a CTG repeat expansion localized in the 3' UTR of the DM protein kinase gene (DMPK). The mechanisms of pathogenesis leading to this multisystemic disorder including myotonia, progressive weakness and wasting of skeletal muscle and extramuscular symptoms such as cataracts, testicular atrophy, endocrine and cognitive dysfunction are complex. Recent reports have revealed that haploinsufficiency of the DMPK gene can account for cardiac conduction defects while cataracts can be explained by haploinsufficiency of the neighboring gene, the DM Associated Homeobox Protein (DMAHP, or Six 5). Furthermore, mice that expressed CUG expansion in an unrelated mRNA developed myotonia and myopathy supporting a RNA gain of function. Our transgenic mice carrying 45 kb of the human genomic DM region with the DMWD, DMPK and SIX5 genes and either 20, 55 or 320 CTG have been used to study if the expression of a human DMPK gene with expanded CTG leads to a DM phenotype. We observed that homozygous transgenic mice carrying a CTG expansion and expressing enough abnormal DMPK mRNA with at least 300 CUG repeats display clinical, histological, molecular and electrophysiological anomalies in skeletal muscle consistent with what is observed in DM1 patients. Furthermore, we observed, as recently described in DM1 patients, a modification in microtubule-associated tau protein expression, which may be involved in the neurological defect in DM1 patients. These results add further evidence of the RNA trans-dominant effect of the mutation causing DM1, not only in muscle but also in brain.

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14. Alternative splicing of DMPK is affected in *cis* in DM1: hints for mechanisms for nuclear retention?

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Myotonic Dystrophy type 1 (DM1) is caused by expansion of a triplet repeat unit in the 3'UTR of DMPK. This has consequences at several levels, one of the most significant being the formation of foci of triplet repeat containing RNA in the nucleus. However, the reason for nuclear retention of expansion-derived transcripts is largely unknown. In order to establish whether specific steps in the processing of these transcripts might be affected by repeat expansion, and whether RNA processing defects could produce the nuclear retention phenomenon, we have been studying the profiles of alternative splicing and polyadenylation of expansion derived transcripts.

DMPK has several alternative splice forms; the frequency of their use and the nature of any effect mediated by the repeat, either in cis or in trans, have not yet been fully investigated. Using affected and control fibroblasts, heterozygous for a SNP in Exon 10 of DMPK, we have been able to develop a fluorescence based quantitative RT-PCR method in order to discriminate between transcripts derived from the affected and unaffected chromosomes. By a combination of dual-colour fluorescence and size discrimination, we have established that the profile of alternative splicing is affected in cis by the myotonic dystrophy triplet repeat expansion.

Although an altered profile of alternative splicing for the expansion allele will not lead to changes in protein isoforms (as the transcripts do not escape the nucleus), the finding that this particular processing event is affected in *cis* may provide some insight into the mechanisms behind the initial cause for nuclear retention.

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15. Mis-splicing of Clc-1 mRNA in a transgenic model of myotonic dystrophy

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Myotonia implies dysfunction of ion channels in the muscle membrane. The exact nature of the channelopathy in DM1, however, is not clear. Intracellular recordings have shown reduced chloride conductance in segments of muscle fibers from DM1 patients (*C Franke et al, J.Physiol., 1990*). However, the abnormality was not consistent or profound, and the relationship to myotonia was uncertain. We explored the basis for myotonia in HSA^{LR} transgenic mice that express expanded CUG repeats. The chloride conductance in HSALR mice was markedly reduced, in a range sufficient to account for myotonia (*M Takahashi and S Cannon, this meeting*). To investigate the mechanism for reduced chloride conductance, we examined the expression of chloride channel 1 (Clc-1), the major chloride channel in skeletal muscle. Mutations which inactivate this channel or alter its gating properties cause hereditary myotonia in mice and humans. The major findings were as follows. (1) The total level of Clc-1 mRNA in HSA^{LR} muscle was slightly reduced (~40% of wild-type). (2) Up to 44% of Clc-1 mRNA in HSALR mice contained a novel exon, exon 7a. By contrast, this exon was included in 6% of Clc-1 mRNA from wild-type mice. Inclusion of this exon causes frame shift and premature truncation at codon 294 (of 994), and these isoforms are not expected to encode functional channel. (3) Mis-splicing of Clc-1 mRNA was not confined to exon 7a. Among 29 cDNAs cloned from HSA^{LR} mice, we observed eleven different alternatively spliced exons. 72% of clones had frame shift resulting in >50% truncation, 28% had more than one aberrant splice, and only 24% encoded full-length Clc-1. (4) In HSA^{LR} mice, antibodies to the carboxyl terminus of Clc-1 revealed a mosaic pattern in which some muscle fibers had a normal circumferential rim of Clc-1 protein, but most were devoid of Clc-1 or had interruptions in the sarcolemmal rim of staining. These results indicate that nuclear accumulation of expanded repeats causes aberrant splicing of Clc-1 mRNA, loss of Clc-1 protein, reduced chloride conductance, and consequently hyperexcitability of the muscle membrane in an animal model of DM1.

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16. Characterizaion of skeletal muscle hyperexcitability in a transgenic mouse model of myotonic dystrophy

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The genetic defect of myotonic dystrophy (DM) is an expanded CTG repeat in the 3' untranslated region of the DM protein kinase gene. Recently transgenic mice which express an untranslated CUG repeat in an unrelated mRNA have been engineered (*Science 289: 1769-1772, 2000*). The skeletal muscle of these mice show membrane hyperexcitability and histological changes which are characteristic of DM, thus suggesting that transcripts with expanded CUG repeats are sufficient to generate the DM phenotype. As the first step to clarify the mechanism by which the transcripts with expanded repeats induce hyperexcitability (myotonia) in these mice, we characterized the physiological basis of muscle hyperexcitability with intracellular recording from dissected intercostal muscle fibers. In fibers from transgenic mice, repetitive action potentials were frequently observed in response to stimulation with current injection. In age-matched controls, the same stimulus elicited only a single action potential. Moreover, the stimulus threshold was lower in fibers from transgenic mice and the latency was prolonged. Membrane time constants measured by applying depolarizing current to ~ -55 mV were significantly slower for transgenic mice (transgenic 17.3 ± 3.0 ms (n = 11), control 4.5 ± 0.4 ms (n = 13)). These changes imply the resting membrane conductance was abnormally low in transgenic fibers. The contribution of Cl- to the resting conductance was measured by switching to a Cl-free bath. Under these conditions, the membrane time constant of control muscle was prolonged 4-fold (showing the conductance had a high dependence on Cl), whereas the time constant of transgenic muscle was only 1.4-fold longer.. The estimated Cl- conductance at ~ –70 mV in transgenic mice was reduced to ~10% of the control (transgenic 59 ± 7.4 (n = 9), control 650 ± 90 μS/cm2 (n = 14)). In conclusion, the hyperexcitability of muscle and myotonia, in a transgenic mouse model of myotonic dystrophy, are due to a reduction of the resting chloride conductance.

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17. Aberrant regulation of Clc-1 chloride channel alternative splicing in DM1 skeletal muscle: a likely cause of myotonia

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A primary feature of myotonic dystrophy (DM1) is myotonia, an electrophysiologic disturbance in skeletal muscle characterized by hyperexcitability of muscle membranes. The molecular basis for myotonia in DM1 is unknown. Loss of function of Clc-1, the predominant chloride channel expressed in skeletal muscle, causes myotonia in humans, goats, dogs, and mice. Here we identify two previously unrecognized alternative exons that are inserted between exons 6 and 7 during splicing of the Clc-1 mRNA. RT-PCR analysis demonstrates that alternative splicing of the Clc-1 mRNA in DM1 skeletal muscle generates four mRNAs, only one of which encodes the full-length chloride channel. The other three mRNAs contain premature termination codons. RNase protection assays demonstrate a >10 fold decrease in Clc-1 mRNA in DM1 skeletal muscle. Immunoblot analysis using anti-peptide antibodies directed against Clc-1 protein demonstrates a corresponding reduction of Clc-1 protein in DM1 skeletal muscle. We conclude that aberrant regulation of Clc-1 alternative splicing generates mRNAs that are degraded by nonsense mediated decay resulting in a loss of Clc-1 function and myotonia.

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18. Involvement of muscleblind proteins in the myotonic dystrophies

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The RNA dominance model proposes a common molecular mechanism as the underlying cause of the myotonic dystrophies. According to this model, DM1, DM2 and possibly DM3 disease is the result of expression of mutant gene transcripts that contain either CUG or CUG-related expansions. These expansions, which fold into highly stable double-strand (ds) RNA hairpins, act as high affinity binding sites for the muscleblind family of dsRNA-binding proteins resulting in the nuclear accumulation of dsRNA-muscleblind protein complexes. DM disease might be due to sequestration of the muscleblind proteins, and the corresponding loss of their normal functions in the nucleus and cytoplasm. Alternatively, the formation of these RNA-protein complexes might impair nuclear function. To distinguish between these possibilities, current work is focused on developing mouse muscleblind knockout lines as well as characterizing the normal functions of the muscleblind proteins in the nucleus and cytoplasm.

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19. Functional analysis of CUG-BP1 and EXP/MBNL

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Myotonic dystrophy (DM) is caused by the expansion of CTG repeat located in the 3' untranslated region (3'UTR) of DM protein kinase (DMPK) gene. Some factors have been reported to interact with CUG repeat in the mRNA and they might be involved in the pathogenesis of DM. We have attempted to analyze two of these factors, CUG-BP1 and EXP/MBNL.

CUG-BP1 is a kind of well-conserved RNA binding proteins, which have several homologs. It has been reported as a splicing enhancer which functions through binding to CUG repeat and the manner of splicing is impaired in the DM cells. In some reports, however, this protein seems to interact with relatively small tract of CUG repeat, not with expanded one. Moreover, we found that CUG-BP1 specifically interacts with UG dinucleotide repeat. EXP/MBNL has been shown to bind double-stranded CUG repeat and colocalize with DMPK mRNA harboring expanded CUG repeat in DM fibroblast cells.

We first studied the effect of CUG-BP1 on UG repeat-containing genes. We cloned 3'UTR of synaptotagmin I, ZnT-3 and 14-3-3 sigma, which have UG repeat in their 3'UTR, in the downstream of luciferase and co-expressed to detect the luciferase activities in the presence/absence of CUG-BP1 and UG repeat. The result indicates iridescent effects of CUG-BP1, as it appears to interact with some reporter genes and fluctuate their activities. But in general, the presence or absence of UG repeats had relatively small influence compared to that of CUG-BP1 on the luciferase activity.

Next, we cloned EXP/MBNL from human skeletal muscle cDNA library and obtained 5 kinds of splice variants including 2 newly identified forms. To compare the affinity to CUG repeat, both CUG-BP1 and the longest form of EXP (EXP42) were assayed in the yeast three-hybrid system. Contrary to the expectation, EXP42 showed little interaction with CUG repeat in this system, while CUG-BP1 had a weak affinity to small number of CUG repeat.

We observed intercellular localization of CUG-BP1 and EXP using immunofluorescence and YFP-tag in COS-7 and C2C12 myoblast cells. CUG-BP1 distributed in both cytoplasm and the nucleus, but the latter was dominant. EXP showed exclusive localization in the nucleus.

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20. *In vivo* co-localisation of MBNL protein with DMPK expanded-repeat transcripts

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Myotonic Dystrophy (DM1) is the most common form of muscular dystrophy affecting adults and is inherited as an autosomal dominant trait. The genetic basis of DM1 is the expansion of a CTG repeat in 3' untranslated region of a protein kinase gene (DMPK). The molecular mechanism by which this expanded repeat produces the pathophysiology of DM1 remains unknown. Transcripts from the expanded allele accumulate as foci in the nucleus of DM1 cells and it has been suggested that these foci sequester cellular proteins that are required for normal nuclear function. We have investigated the role of three proteins, CUG-BP, hnRNP C and MBNL as possible sequestered factors. Using a combination of indirect immunofluorescence to detect endogenous proteins, and overexpression of proteins with GFP tags we have shown that CUG-BP and hnRNP C do not co-localise with expanded repeat foci in DM1 cell lines. However, GFP tagged MBNL does appear as foci in DM1 cell lines and co-localises with expanded repeat transcripts. GFP tagged MBNL does not appear as foci in non-DM1 cell lines. This work provides further support for the involvement of MBNL in DM1.

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21. Molecular basis for impaired differentiation in myotonic dystrophy

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Differentiation of skeletal muscle is affected in Myotonic Dystrophy (DM) patients. Analysis of cultured myoblasts from DM patients shows that DM myoblasts lost the capability to withdraw the cell cycle during differentiation. Our data demonstrate that the expression and activity of the proteins responsible for cell cycle withdrawal are altered in DM muscle cells. E2F/Rb and E2F/p130 repressor complexes are required for cell cycle withdrawal and are abundant in differentiated cells from normal patients. On the contrary, DM muscle cells do not form Rb/E2F repressor complexes. The formation of Rb/E2F complexes in skeletal muscle cells is controlled by cdk4. We found that, the activity of cdk4 declines during differentiation of muscle cells from control patients, while in DM cells cdk4 is highly active during all stages of differentiation leading to hyper-phosphorylation of Rb and to the block in the formation of Rb/E2F complexes. Investigations of the regulators of cdk4 activity in DM and in control cells show that DM cells fail to induce protein levels of p21, a key negative regulator of cdk4 activity. Further studies of p21 regulation in DM cells demonstrated that p21 mRNA is induced in both DM and normal cells during differentiation; however, translation of p21 mRNA is reduced in DM cells. We found that skeletal muscle cells from DM patients fail to induce cytoplasmic levels of a CUG RNA binding protein, CUGBP1, while normal differentiated cells accumulate CUGBP1 in the cytoplasm. In muscle cells from normal patients, CUGBP1 up-regulates p21 protein during differentiation. Several lines of evidence show that CUGBP1 induces translation of p21 via binding to a GC rich sequence located within the 5' region of p21 mRNA. We suggest that the failure of DM cells to accumulate CUGBP1 in the cytoplasm leads to a significant reduction of p21 and to alterations of other proteins responsible for the cell cycle withdrawal, such as cdk4 and Rb. These data provide evidence for an impaired cell cycle withdrawal in DM muscle cells and suggest that alteration in activity of CUGBP1 causes disruption of p21-dependent control of cell cycle arrest.

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22. Identification of the CUG repeats binding proteins in HeLa and myoblast

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One key area in Myotonic Dystrophy is defining the CUG binding protein factors to explain the foci formation in patient's Myoblasts. Although several factors have been reported, we investigated additional factors by adopting a different extract preparation method and a modified UV cross-linking assay. We use 5, 46 and 85 repeats of CUG RNA and look for proteins which do not bind the (CUG)5 RNA, but have a stronger affinity for the 85 versus the 46 in HeLa cell extracts. We found cross-linking bands that are common the (CUG)46 and 85, but show much stronger binding affinity to the (CUG)85. A protein has been purified from a CUG affinity column. This will be sequenced and further characterized. Biochemical data suggest that the protein can be multimerized depend on the size of CUG repeats.

A parallel approach was taken using a DM patient's Myoblast extract. We found two cross-linking products, which are specific to the 46 and 85 repeats. The purified protein sample indicates that the proteins form different cross-linking products depending on the length of the repeats. We are currently trying to understand the relationship between the CUG repeats and the binding proteins from the HeLa and Myoblast extract.

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23. Characterization of monoclonal antibodies against DMPK and application to changes in DMPK levels during development and in myotonic dystrophy

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We have used a panel of monoclonal antibodies (mAbs) against the myotonic dystrophy protein kinase, DMPK, to show that this is an 80-85kD protein whose expression is essentially restricted to skeletal and cardiac muscles. Other proteins recognized by some of the mAbs, including a 72kD protein, are shown to be cross-reacting protein kinases.

The levels of expression of 85kD DMPK protein have been determined at different stages of human development in normal and DM skeletal muscle. DMPK is detected in skeletal muscle between 11 and 18 weeks of foetal development and thereafter remains at high levels into the neonatal period, but levels were 60% lower in adult muscle. Consistent with this, a 15-fold increase in DMPK was observed during human myoblast differentiation in cell culture. DMPK levels were compared between normal foetuses and age-matched foetuses with severe congenital myotonic dystrophy. DMPK in quadriceps of DM-1 patients with large CTG (> 1500) was reduced to 65% of control levels. The results are consistent with greatly reduced expression from the mutant allele and normal expression from the unaffected allele.

Supported by grants from Association Française contre les Myopathies (AFM) and the Muscular Dystrophy Campaign (UK).

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24. C-terminal and internal VSGGG peptide motifs confer distinct properties to myotonic dystrophy protein kinase (DMPK) isoforms

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It is well accepted that the time of onset and clinical appearance of myotonic dystrophy (DM) globally correlates with the length of the $(CTG)_n$ tract in the 3'-untranslated region of the Myotonic Dystrophy Protein Kinase (DMPK) gene. However, the molecular mechanism by which $(CTG)_n$ expansion contributes to disease aetiology remains elusive. In our studies we have focussed on the role of DMPK, a member of the family of Rho-binding Ser/Thr kinases, and a likely candidate to be involved in DM pathology. RT-PCR experiments show that three major splice events generate six mature DMPK mRNA isoforms [*Groenen et al., Hum. Mol. Genet. 9:605-616, 2000*]. In both man and mouse, the alternative splicing results in protein products with three different (tail-less, hydrophobic or less-hydrophobic) C-termini and in presence or absence of an internal VSGGG peptide sequence. Two C-terminally truncated ~68 kDa DMPK isoforms are mainly expressed in smooth muscle, the other four ~74 kDa DMPK isoforms are heart, skeletal muscle, or brain specific. Both $(CTG)_n$ repeat expansion as well as (trans-acting or intrinsic?) kinase activity may alter DMPK isoform composition and activity in this complex. We are, therefore, currently trying to establish a better picture of the role of the splice and post-translational modifications in DMPK proteins. Here we report on the study of the biochemical properties and cellular routing behaviour of each of the six different isoforms. Transfection into COS1, neuroblastoma N2A and 3T3 fibroblast cells and confocal laser scanning microscopy showed that the DMPK isoforms with the hydrophobic C-terminal domain are distributed in a reticular-like fashion throughout the cytoplasm. The tail-less variant shows a smooth cytosolic distribution, whereas presence of the less-hydrophobic C-terminus directs the protein to a distinct organellar location. A fraction of the VSGGG containing DMPK isoforms show a minor shift towards a higher molecular mass. This shift is absent in DMPK kinase-dead isoforms, suggesting a role for the VSGGG moiety in post-translational (auto)phosphorylation-modification of DMPK. Finally, yeast two-hybrid and immune pull–down experiments suggest that DMPK has a role in a larger protein assembly in which also myosin light chain, and myosin-light chain phosphatase play a role.

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25. Expression of DMPK in the autopsied skeletal muscle with severe congenital myotonic dystrophy (cDM)

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BACKGROUND: cDM (congenital myotonic dystrophy) patients have been well known to have quite different clinical features from adult DM. However, conflicting results on cDM studies of mRNA and DMPK expression have been reported.
OBJECTIVE: To report the expression of DMPK in autopsied skeletal muscle with severe cDM.
METHODS: Southern blot analysis of (CTG)n repeats of DMPK gene and histochemical studies were performed on six autopsied cases of cDM, who died within the age of one year, compared with controls.
RESULTS: DMPK was expressed in only large muscle cells before birth and the expression of almost adult pattern, which shows mosaic pattern with tree different intensities, was observed in 2 year-old skeletal muscle, although the diameter of each muscle fiber was quite smaller than that of adult one. The muscle of the most severe cDM floppy infants, who died at birth with respiratory distress and no muscle movement, showed quite immature and the expression of DMPK mainly expressed in large round muscle fibers looking like the control muscle obtained from 28-week fetus. However, in severe cDM, who had muscle movement, but died at the age of 11 months, mosaic pattern of DMPK expression with three different intensities was observed similar to two- year-old skeletal muscle. The diameter of each muscle fiber was quite smaller than that of normal two-year-old and adult ones. Interestingly, two groups of severe cDM patients, which died at birth (two patients) and 11 month (two patients) had almost the same size of expanded (CTG)n repeats in their muscles (15~18 kb, 15~17 kb, respectively), but the expression of DMPK was quite different.
CONCLUSION: Our studies indicate that DMPK is expressed even in most severe cDM skeletal muscle, and DMPK expression is developmentally regulated even after birth.

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26. Ubiquilin-1 binds to myotonic dystrophy protein kinase (DMPK) and modulates its kinase activity and degradation

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An expansion of the CTG repeat in the 3'-untranslated region of the *DMPK* gene causes myotonic dystrophy type 1. DMPK is one of the serine-threonine kinases belonging to the Rho kinase family, and has been shown to phosphorylate dihydropyridine receptor, phospholemman, and myosin phasphatase subunit-1 (MYPT-1). A small heat shock protein designated as "DMPK-binding protein (MKBP)" regulates the kinase activity of DMPK. Although DMPK appears to regulate cell size and shape and modulate the Ca^{2+} homeostasis of skeletal muscle, the exact physiological functions of this kinase remain to be elucidated.

In this study, we used the full-length DMPK as the bait in the yeast two-hybrid screening, and identified an ubiquitin-like protein, ubiquilin-1, as an interacting protein. Ubiquilin-1shows 99% homology to hPLIC1, which has been postulated to provide a link between the ubiquitination machinery and the proteasome. *In vitro* binding assay confirmed the interaction, and demonstrated that the Stil-like repeat element domain of ubiquilin-1 is required to bind to the kinase domain of DMPK. The interaction was also demonstrated in a mammalian cell line, Cos-1. Colocalization of both proteins was immunohistologically demonstrated in the cytoplasm and, possibly in the nucleus, of Cos-1 and HEK293 cells that were transfected with cmyc-DMPK and FLAG-ubiquilin-1. DMPK did not phosphorylate ubiquilin-1; however, the equimolar or a lower amount of ubiquilin-1 (0.5-50 nM) increased the kinase activity of DMPK three to four-fold, whereas higher concentrations (0.5 – 2.0 μM) inhibited the DMPK transphosphorylation of a small MYPT-1 peptide. The half-life of DMPK determined by pulse-chase experiments using ^{35}S-methionine was ~43 hours, which was prolonged to ~57 hours by co-transfection of ubiquilin-1, suggesting that ubiquilin-1 inhibits the degradation of DMPK. Our results indicate that ubiquilin-1 may be a chaperone protein that can modulate DMPK protein folding and/or targeting.

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27. Regulation of serum response factor by myotonic dystrophy protein kinase (DMPK)

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The transformation of replicating myoblasts into terminally differentiated myotubes is a critical process in myogenesis. Serum response factor (SRF), a transcription factor essential for both early mesodermal development and late myogenic differentiation, is known to play a key role in this process, but the mechanisms are unclear.

We have found that myotonic dystrophy protein kinase (DMPK) enhances SRF-mediated *c-fos*, skeletal α-actin and cardiac α-actin gene transcription in COS cells and C2C12 myoblasts. DMPK also synergizes with activated RhoA to enhance SRF-mediated *c-fos* transcription. DMPK phosphorylates SRF stoichiometrically in the "MADS box", a highly-conserved region required for DNA binding, dimerization and co-activator interaction. Detailed phosphopeptide mapping reveals that DMPK targets a peptide in the αI coil in the DNA-binding domain of SRF both *in vitro* and *in vivo*. Threonine 159 and serine 162 within this peptide are specifically phosphorylated *in vitro*. Substitution of Thr159 by aspartic acid results in a small increase in SRF-DNA binding as well as target gene transcription, whereas substitution of Ser162 with aspartic acid results in diminution of both DNA binding and transcriptional activity. SRF also associates physically with DMPK, both *in vitro* and *in vivo*.

DMPK is likely to be a direct regulator of SRF, enhancing SRF-mediated gene transcription partly by phosphorylating Thr159 in the αI coil of the MADS box. Differential phosphorylation of key residues in this region (Thr159 vs. Ser162) could constitute an important regulatory mechanism for up- or down-regulating myogenic differentiation.

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28. DMPK:Regulator of cytoskeletal – membrane dynamics

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Myotonic Dystrophy Protein Kinase (DMPK) is encoded by the Myotonic Dystrophy 1 (DM1) locus. Studies on human tissues and cultured cells suggest that haploinsuffirency affecting DMPK expression is a significant consequence of the dCTG repeat expansion mutations that cause DM1. DMPK is also representative of a group of serine-threonine protein kinases active in cell-cycle control, cell differentiation, and cytoskeletal organization. At least three other human protein kinases are also members. Studies in our laboratory suggest as a nexus between the distinct signaling networks containing Rac-1 and Raf-1 kinase. DMPK also converges with Rho kinase in the regulatory inactivation of myosin phosphatase, a key control element for the actin cytoskeleton. DMPK along with Rac-1, Raf-1 kinase, and myosin light chain kinase (MLCK) may represent a membrane-associated signaling system whereas Rho kinase and RhoA would be primarily a cytosolic pathway for regulating the assembly and function of cytoskeletal myosin II and actin.
Supported by the MDA, NINDS, and NHLBI.

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29. DMPK in for synaptic plasticity of the hippocampus

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Patients with early onset myotonic dystrophy (DM) show frank mental retardation while those with later-onset disease show decreased IQ. These findings suggest a role for DMPK in CNS function. To gain insight into that role, we examined synaptic transmission and plasticity in the myotonic dystrophy protein kinase (DMPK) knockout mouse. Synaptic transmission and long-term synaptic potentiation (LTP) were normal; however, there was a significant decrease in the decremental phase of potentiation, with a duration of several hours, that accompanies LTP. These finding supports a role the DMPK in synaptic plasticity, and suggests a possible mechanism for cognitive dysfunction in DM. The selective loss of decremental potentiation also suggests that it may be expressed through different synaptic mechanisms than LTP.

Supported by the MDA, NINDS, Hunter and Vasku Funds.

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30. Abnormal cardiac Na channel gating in mice lacking DMPK

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Skeletal muscle excitability is more affected than cardiac muscle excitability in DM, and skeletal muscle Na channels are more affected by co-expressed DMPK in *Xenopus* oocytes than the cardiac isoform. Cardiac conduction defects, particularly delayed conduction and heart block, are nonetheless common in patients with DM. Previously, we found that skeletal muscle Na channels in mice partially or completely deficient in DMPK had more late re-openings, an abnormality that led to repeated action potentials and might contribute to muscle weakness. We now find similar results in cardiac muscle Na channels.

We enzymatically isolated ventricular myocytes from DMPK+/+ (wild-type), DMPK+/-, and DMPK-/- mice using a Langendorff perfusion technique and used patch clamp methods to measure Na currents. In macroscopic currents measured in whole-cell recordings, we found no reduction in current density, no changes in equilibrium measures of activation and inactivation gating, and no changes in recovery from or development of inactivation. In microscopic currents measured in cell-attached patches from DMPK+/- and DMPK-/- mouse hearts, we found an increased proportion of traces with late Na channel re-openings. We quantified this finding using $P(NP_o>0.1)$, the proportion of traces with probability of opening more than 10% between 10 and 110 msec following the voltage step. This measure was increased from 0.028 in wild-type (n=6 patches) to 0.078 in DMPK+/- (n=4) and to 0.104 in DMPK-/- mice (n=4).

We conclude that DMPK+/- and DMPK-/- mice have the same phenotype in which:

1. like skeletal muscle Na channels, cardiac Na channels re-open
2. unlike skeletal muscle Na channels, there is no reduction in cardiac Na current density

We suggest that DMPK modulates Na channels at more than one site. The effect of DMPK deficiency to allow re-openings in both skeletal and cardiac Na channels may be mediated through a highly-conserved site such as the channel inactivation gate, while the effect to reduce skeletal but not cardiac muscle Na current density may be mediated through a channel isoform-specific site.

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31. Expression of human DM protein kinase (DMPK) in *Schizosaccharomyces pombe*

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It has been indicated that, Myotonic Dystrophy Protein Kinase (DMPK) affects intracellular cytoskeletal systems like Rho kinase, although DMPK lacks apparent Rho-binding domains. We expressed human DM protein kinase in fission yeast *Schizosaccharomyces pombe*, of which gene expression and cell morphology is well studied. Human DMPK constructs are ligated into yeast expression vector (*nmt*1 promoter), and protein expression was induced by removing thiamine from the medium.

Overexpression of human DMPK affected cell viability and cell shape. C-terminus deleted DMPK showed the middle-swollen phenotype (lemon-like shape), indicating cell septum abnormality. On the other hand, when both kinase domain and C-terminus is present, the expression of DMPK resulted in polarized cell growth and multi-septum/blanched cell. We did not observe any morphological changes by expressing only kinase domain of DMPK.

A lemon-like phenotype of DMPK without C-terminus disappeared by disrupting ATP binding site of DMPK, exchanging lysine to arginine (K100R mutant). However, polarized and/or multi-septum cells lacking DMPK N-terminus were not rescued by K100R mutation.

Recombinant DMPK proteins with C-terminus were recovered as an insoluble protein after centrifugation at 10,000×g. On the other hand, constructs lacking C-terminus were soluble.

Taken them together, we conclude that the N-terminus of DMPK plays an important role in the DMPK kinase activity, and the C-terminus of DMPK determines intracellular localization of this protein. C-terminus of DMPK may also have a function as a negative regulator of DMPK activity.

Interestingly, both multi-septum and lemon-shape cell are typical abnormal phenotypes of Rho overexpression and F-actin associated protein (Pob1p) disruption in *S. pombe*. Our study indicates that DMPK contributes to Rho-induced cytoskeletal reorganization.

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V. Molecular mechanisms of insulin resistance

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Insulin mediates its glucose lowering effect by activating tyrosine kinase activity of insulin reveptor, tyrosine phosphorylating insulin receptor substrates(IRSs) such as IRS-1 and IRS-2, thereby activation PI3 kinase, leading to stimulation of glucose transport and glycogen synthesis in the skeletal muscle as well as inhibition of glucose output from the liver. Mutaion of the insulin receptor cause insulin resistance and diabetes in humans and mice[1]. Targeted disruption of IRS-1 causes insulin resistance compensated by β cell hyperplasia and features of syndrome X such as hypertriglyceridemia and hypertention[2]. Targeted disruption of IRS-2 causes insulin resistance not compensated by β cell hyperplasia, thereby causing diabetes[3].

Obesity is characterized by hypertrophic adipocytes whitch secrete excess amount of TNF-α, FFA, resistin and decreased amount of adiponectin, thereby collectively causing increased tissue triglyceride content in the skeletal muscle and liver associated with serine phosphorylation of IRS-1 and IRS-2, leading to insulin resistance[4-6]. Mutations of adipogenic transcriptional factors and their coactivators such PPARγ[7,8] and CBP[9] prevent from adipocyte hypertrophy and cause increased adiponectin & leptin, thereby protecting against obesity-induced insulin resistance. SNPs of human PPARγ and adiponectin genes confer susceptibility / resistance to obesity and diabetes.

Thus, genetically determined and life-style-related defects in insulin signalling pathways are associated with insulin resistance, obesity, features of syndrome X and type 2 diabetes.

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VI. Regulation of muscle cell differentiation, growth and apoptosis

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I will review a series of investigations that have explored the molecular mechanisms controlling skeletal muscle cell differentiation and growth. Myogenic precursor cells, referred to as myoblasts, irreversibly withdraw from the cell cycle as they differentiate into mature myotubes. During in vitro myogenesis a portion of myoblasts undergo apoptosis while others continue with their differentiation program and form myotubes that are more resistant to cell death. Presumably, the coordinated regulation of cell proliferation and apoptosis provides the embryo with a mechanism to limit the deposition of muscle mass during development. Studies have shown that the upregulation of the cdk inhibitor p21 and the dephosphorylation of pRb appear to be critical regulatory events for the establishing both the post-mitotic and apoptosis-resistant states during myogenic differentiation. More recently, it has been shown that cell cycle withdrawal during myogenesis facilitates the induction of the Akt/PKB protein kinase which, in turn, contributes to enhanced myocyte survival. Further studies have shown that Akt signaling also plays an important role in skeletal muscle maturation by regulating myofiber hypertrophy and blood vessel recruitment.

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32. A panel of monoclonal antibodies against human Six5 identifies a 100kD nuclear protein in human cell lines and tissues

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Six5 is one member of the Six family of homeobox-containing transcription factors involved in eye development. In DM, the expanded CTG repeat lies within the 5'-UTR of the Six5 gene (once known as DM-associated homeobox protein, DMAHP). Like myotonic dystrophy patients, Six5 knockout mice develop cataracts but Six5 mRNA has a wider tissue distribution, suggesting some role for Six5 protein in other tissues. Six5 protein is produced from three exons, A, B and C, and exon A contains the homeobox sequence, the conserved Six domain and the nuclear localization signal.

We cloned exons A and B separately into a bacterial expression plasmid and used the recombinant proteins obtained to immunize Balb/c mice for hybridoma production. Transfection of HeLa cells with full-length Six5 in pCDNA4 results in localization to the nucleus and we screened hybridomas for their ability to recognize the nuclear recombinant Six5 in transfected cells. Ten mAbs against exon A and four mAbs against exon B were obtained and cloned twice to homogeneity. The availability of mAbs against at least two different regions of Six5 enables conclusive identification of authentic Six5 as a protein recognized by all mAbs.

Preliminary studies suggest that Six5 migrates on SDS-PAGE as a 100kD protein, slightly larger than predicted from the amino-acid sequence and recognized by all 14 mAbs. The mAbs detect endogenous Six5 in nuclei by immunofluorescence microscopy and its distribution within the nucleus is similar to that of transfected Six5. The tissue distribution of the protein has been studied.

Supported by grants from the Muscular Dystrophy Campaign (UK).

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33. The *Drosophila* homologue of the myotonic dystrophy associated gene, *SIX5*, is required for muscle and gonad development

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Myotonic dystrophy type 1 (DM1) is characterised by a number of pathologies including muscle defects and testicular atrophy. The mutation causing the disease is a CTG triplet repeat expansion in the 3' untranslated region of a protein kinase gene, *DMPK*, and the promoter region of a homeobox gene, *SIX5*. The mechanism by which the mutation causes the disease phenotype still has to be elucidated, but the *SIX5* transcript has been shown in several studies to be reduced in DM patients. *SIX5* is a member of the *Six* family of genes, named after the first member found in *Drosophila melanogaster*, *sine oculis*. We show that *DSix4* is the closest *Drosophila* homologue of *SIX5* and characterise the wild type expression pattern. Our findings show that *DSix4* is involved in mesoderm, gonad head and central nervous system patterning in the developing embryo. Moreover, adult males with a defective *D-Six4* gene exhibit testicular atrophy. The defects in *D-Six4* mutant flies suggest that human *SIX5* should be more strongly considered as responsible for the muscle wasting and testicular atrophy phenotypes in DM1.

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34. The murine *Six5* locus is essential for gametogenesis

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Work in progress

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35. Involvement of SIX5 in DM pathogenesis

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SIX5 gene is one of the members of homeobox gene family SIX and thought to be involved in the DM pathogenesis through the diminished expression level leading to the abnormal level of expression of its target genes. To understand how SIX5 is involved in DM pathogenesis, we are taking three lines of approach. First, we are analyzing mice defective of both Six4 and Six5 genes and look for phenotype in muscle and other tissues. Second, we are identifying Six5 target genes and examining the regulatory mechanism by Six5 protein. Third, we are studying cooperative action among Six, Eya and Dach proteins, which is a conserved for the fly compound eye formation and the vertebrate muscle formation. Very recently, Drosophila homologue of SIX5, D-Six4, is shown to be involved in muscle and the mesodermal component of gonad formation (*Kirby et al. Curr. Biol. 2001*). Considering the genetic and functional conservation of Six family genes in various species, it is reasonable to propose that SIX4/SIX5 genes are involved in muscle and gonad formation in human. Although Six5 knockout mice showed little phenotype in muscle (*Klesert et al, Nat. Genet 2000, Sarkar et al, ibid 2000*) and Six4 knockout mice also showed no apparent phenotype, functional redundancy between Six4 and Six5 is suggested (*Ozaki et al. Mol. Cell. Biol. 2001*). We established Six4/Six5 double knockout mice and more than 50% of the Six4-/-Six5-/- offspring died within a few hours after birth. We are currently examining the detailed phenotype of the double knockout mice. Recent report indicated that the Six-Eya-Dach gene network induced muscle differentiation in chick cultured myoblast (*Heanue et al. Genes. Dev. 1999*). In fact, Eya had been shown to act as a co-activator of Six5 in the activation of myogenin gene transcription through nuclear translocation and complex formation with Six (*Ohto et al. Mo. Cell. Biol. 1999*). In this context, the mechanism of action of a transcription factor Six5 should be clarified in the context of cooperative interaction with Eya and also with Dach. We performed interaction studies with Six and Eya and also with Eya and Dach by GST-pull down, mammalian two-hybrid and co-immunoprecipitation analyses. The interaction between Eya and Dach is enhanced in the presence of Six5.

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36. Transcriptional targets of SIX5

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SIX5, encoding a homeodomain transcription factor, is expressed in most tissues affected by DM1 such as skeletal and smooth muscle, central and peripheral nervous system and adult lens. We hypothesized that *SIX5* deficiency, in conjunction with other molecular defects resulting from the CTG repeat expansion, is involved not only in the development of cataract but also in the development of neuromuscular symptoms found in DM1 patients. In this study, we sought to identify transcriptional targets for SIX5 to understand the molecular mechanism by which SIX5 deficiency causes cataract and to relate SIX5 deficiency with other DM1 symptoms.
To identify target genes whose expression are directly regulated by SIX5, we overexpressed a constitutively active form of mouse Six5 fused to a potent transcription activation domain of VP16 (VP16-Six5wt) in several different cell types by adenovirus-mediated gene transfer and searched for potential target genes upregulated by the treatment. A homeodomain mutation protein VP16-Six5W241R, which shows a defective DNA-binding capacity, was used as a control.
In our initial screening attempt with P19 embryonal carcinoma cells, we found several genes induced by VP16-Six5wt such as genes encoding signaling molecules [Wnt11, Igf2 and Igf binding protein 5 (Igfbp5)], transcription factors (Mdfi, Six2, Six5) and axonal guidance molecules (Sema3C, Sema4C). To verify that genes identified in our screen are directly regulated through Six5-binding sites, regulatory sequences flanking *Igfbp5* were analyzed. In addition, the effect of the absence of *Six5* on *Igfbp5* expression was assessed using embryonic fibroblasts derived from Six5-deficient mouse. We will also present the data obtained from VP16-Six5wt overexpression in C2C12 myoblasts and SRA 01/04 human lens epithelial cells.

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37. Does "painting" of chromosome 19 by (CUG)n-containing DMPK mRNA cause the multisystem phenotype of myotonic dystrophy? A new hypothesis of long-range *cis* autosomal inactivation

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Myotonic dystrophy (DM) is caused by an expanded trinucleotide repeat in the 3' untranslated region of the mRNA of DM protein kinase (DMPK). After many investigations, it appears that the DM "syndrome" (cardiacconduction defects, myotonia, cataracts, diabetes, etc.) is a result of diverse mechanisms influencing different genes: (i) effects of the repeat on the net expression of protein of DMPK, (ii) effects of the trinucleotide-repeat-containing mRNA itself on cell or nuclear functions, and (iii) effects of the repeat on the chromosomal structure in this region. This presentation addresses a further potential mechanism: long-range effects of the DM disease locus on a distant gene, specifically FCGRT. The FCGRT gene encodes the heavy chain of FcRB, the IgG "protection receptor" expressed in vascular endothelial cells that rescues endocytosed IgG antibody from catabolism. The protection mechanism is depressed in DM, compatible with underexpression of the FcRB receptor, leading to selective hypercatabolism of IgG in DM patients. FCGRT has been mapped to the same chromosomal band (19q13.3) as the DM locus, but it is 4 megabases distant. The long-range effects may be confined to the FCGRT allele on the same chromosome (*cis*) or may be experienced by FCGRT on both chromosomes (*trans*) in affected individuals. The degree of loss of IgG protection is compatible with half as much expression of FCGRT, that would be compatible with a monoallelic suppression. The presence of the DM and FCGRT genes in the same chromosomal band suggests that their colocalization may be more than coincidental ($p=0.0003$), i.e., that there is a *cis* mechanism by which DM suppresses the FCGRT gene. If the mechanism is *cis*, it implies an unusual and perhaps novel type of long-range interaction on the DM chromosome, such as RNA "painting" in analogy to *Xist*-induced X chromosome inactivation. Understanding the means of FCGRT suppression in DM may suggest new directions of research by which the multisystem/multigene phenotype of DM may be explained.

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38. Executive-frontal lobe dysfunction in proximal myotonic myopathy (PROMM): evidence for dopamine modulation of limbic-prefrontal cortex circuits?

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Impaired executive functions, apathy, and impulsivity are hallmarks of frontal-subcortical circuit dysfunction. The dopaminergic modulation (D1) of the prefrontal-subcortical (PFC) circuits suggest that this neurotransmitter may play a role in the modulatory influence of limbic-PFC circuits during behaviour. The aim of our study is to correlate the selective impairment in executive-frontal abilities previously demonstrated by neuropsychological and neuropsychiatric investigations in PROMM and DM1 with brain PET scan findings using specific dopaminergic ligands. 25 patients with PROMM, 25 patients with moderately severe DM1 (CTG expansion range = E2) and 25 age, sex and education-matched controls were subjected to: Cognitive test measures: Computerized Attentional Assessment, TEA; Winsconsin Card Sorting Test, WCST; Stroop Test, ST; Trail Making Test A and B, TMT and; Tower of London Test, TLT computerized version; Behavioural test measures: SCID-II personality scale, self-administered anxiety and depression scales, neuropsychiatric interview. Basic attentional and alertness functions (TEA, TMT) were normal in both PROMM and DM1 patients. Cognitive strategies and visual-spatial decisions (WCST, ST, TLT) were significantly impaired in patients with PROMM and DM1 ($p < 0.001$). These cognitive abnormalities do not appear to be attributable to motor impairment in these two cohorts of patients, as many of the cognitive measures, which significantly differentiated the two groups of patients compared to controls, had a minimal motor component. None of our patients fulfilled DSM-IV criteria for axis I and II disorders. Both patients with PROMM and DM1 showed significant avoidant, dependent, obsessive-compulsive behavioural trait clustering ($p < 0.05$). Our data confirm a selective dysfunction of the prefrontal subcortical circuits in PROMM like in DM1. On-going studies with dopaminergic ligands will confirm or refute our hypothesis that dopaminergic signalling in the prefrontal cortex may contribute to the impairment of executive frontal lobe functioning, affecting the planning, initiation and regulation of behaviour in PROMM and DM1. Furthermore, these results may be useful end-point measures in controlled clinical trials in DM1, DM2/PROMM.

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39. Microarray-based expression profiling of gene expression changes in DM1 and DM2

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The myotonic dystrophies (DM) are now collectively recognized as a clinically and genetically heterogeneous group of neuromuscular disorders, characterized by autosomal dominant inheritance, muscular dystrophy, myotonia, and multi-system involvement. For DM1, the variable phenotype and anticipation have been linked to a (CTG)n repeat expansion in the DM protein kinase (*DMPK*) gene in 19q13.3. DM2 maps to a locus in 3q21.3; the gene and its mutation(s) are currently unknown. Given the similar pathophysiological manifestations of DM1 and DM2, we hypothesized that they share a common pathological pathway(s). Similar to DM1, we suggest that DM2 could be due to the expansion of an unstable repeat that affects RNA metabolism, or mutation(s) in a gene involved in RNA metabolism. To test these hypotheses, we used gene expression profiling with microarrays. We globally compared expression for 6,606 non-redundant cDNAs and ESTs (HuGeneFL, Affymetrix, Santa Clara, CA), in skeletal muscle biopsies and muscle cell lines of normal, DM1 and DM2 individuals. Comparison of expression profiles of a pool of 10 normal skeletal muscle biopsies with six DM1 and one DM2 (with confirmed linkage to the 3q21 region) showed considerable overlap in the genes down-regulated among DM1 and DM2 patients and dysregulation of numerous skeletal muscle markers (e.g., myosin heavy chain, α-tropomyosin, desmin, several troponins, as well as α-sarcoglycan, but not dystrophin). Similar changes were seen *in vitro* with primary muscle cell lines from skeletal muscle of DM1 patients – for either total RNA or nuclear and cytoplasmic fractions separated. When DM1 patient myoblasts reach ~70% confluency and are induced to differentiate, they display a block in differentiation into multinucleated myotubes as evidenced by the lack of muscle differentiation markers such as myosin heavy chain or desmin. Moreover, separate expression profiles of nuclear and cytoplasmic fractions, both in myoblasts and differentiated myotubes, indicated a block in nuclear transport for certain genes, such as the skeletal muscle myosin heavy chain 3 (*MYH3*). Statistical analysis verified significant differences between nuclear and cytoplasmic fractions between DM1 patients and normal controls for muscle proteins ($p = 0.052$) and proteins involved in RNA metabolism ($p = 0.003$), indicating a block in nuclear-cytoplasmic export, including DMPK. Taken together, these data suggest a global trans-effect of the DM1-associated (CTG)n expansion. Thus, we propose that the nuclear accumulation of mutant DMPK transcripts with (CUG)n expansions causes a global defect in RNA processing in trans, which is reflected *in vitro* by the inability of the DM1 myoblasts to differentiate normally into multinucleated myotubes. As the cellular pathologies show considerable overlap among DM1 and DM2, expression profiling of the two diseases will provide new and valuable insights into the pathology of DM.

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40. Proximal myotonic dystrophy (PDM) and progressive myotonic myopathy (PROMM) in Finnish and Italian kindreds: linkage to and linkage disequilibrium at the *DM2* locus in 3q21.3 in two separate founder populations

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Myotonic dystrophy (DM 1 and DM2), proximal myotonic myopathy (PROMM), proximal myotonic dystrophy (PDM), and related proximal myopathic syndromes are dominantly inherited, multi-systemic myotonic myopathies characterized by predominantly distal (DM1 and DM2) or proximal (PROMM and PDM) muscle involvement. DM1 is caused by a (CTG)n repeat expansion in the DM protein kinase (*DMPK*) gene in 19q13.3. DM2 maps to a locus in 3q21.3; the gene and its mutation(s) are currently unknown. We previously reported linkage of our original Finnish PDM family and no linkage of one Italian PROMM kindred to the 3q21.3/*DM2* region. We have now identified additional kindreds from both populations (two Finnish, and two Italian kindreds), all of which show linkage to the same 3q21.3 region (Z_{max} = 12.52 for *D3S3584* at θ = 0.005). Using GENEHUNTER, a maximum multi-point lod score (HLOD) of 10.38 was obtained for all five kindreds at α (proportion of linked families) = 1.000. With SIMPLE, a maximum multi-point lod score of 15.38 for the region between *D3S3584* (143.94 cM) and *D3S1292* (146.60 cM) was determined. Further fine mapping of the pedigrees identified shared haplotypes among affecteds consistent with linkage disequilibrium (LD). In each population the disease phenotype segregates on a distinct haplotype, indicating separate founder mutations in the respective populations. By determining the smallest region of overlap, we have further narrowed the critical interval for *DM2* to a region less than 100 kb in 3q21.3. Interestingly, all of our 3q21.3/*DM2*-linked kindreds show predominantly proximal, whereas the family initially used to map DM2 shows predominantly distal muscle involvement. Moreover, the clinical manifestations between each of the linked Finnish kindreds are pronounced, also compared to other PROMM or DM2 pedigrees showing linkage to the same 3q21.3 region. Thus, in spite of shared haplotypes and presumably the same mutations in the Finnish and Italian populations, there is significant clinical heterogeneity.

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41. Molecular genetics of DM2 clarify pathogenic mechanism(s) of myotonic dystrophy

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Myotonic dystrophy (DM) can be caused by a mutation on chromosome 19 (DM1) or 3 (DM2/PROMM). To clarify the pathogenic mechanism of DM, we have identified a second human mutation that causes the same multisystemic features of DM1 affecting skeletal muscle, heart, eye and the endocrine system. Transmission disequilibrium testing and analysis of conserved ancestral haplotypes was used to narrow the DM2 locus to ~320 kb. One of the markers in linkage disequilibrium with DM2, *CL3N58* ($p \leq 0.000001$), showed an aberrant segregation pattern. Southern analysis demonstrated that affected individuals in six DM2 families (LOD=31.6 at Θ=0.00) had an expanded allele too large to amplify by PCR that was not found in controls (n=1360). The sequence of the *CL3N58* marker contains the complex repeat motif $(TG)_n(TCTG)_n(CCTG)_n$. In our controls, the size of the combined $(TG)_n(TCTG)_n(CCTG)_n$ repeat tract ranged from 104-176bp (Het=0.89). Smaller expansions from three DM2 patients were cloned and sequenced, demonstrating that it is the CCTG portion of the repeat tract that is expanded on affected alleles. In contrast to alleles from the control samples, the CCTG repeat tracts on these expanded alleles were uninterrupted. The range of expanded allele sizes is extremely broad (~75-11,000 CCTGs, mean ~5,000) with a high degree of somatic mosaicism. Because shorter expansions were found in individuals with multiple allele sizes in blood, the smallest pathogenic size is uncertain. Surprisingly, the repeat tract continues to increase with age and there is a tendency for offspring to have smaller repeat tracts than their affected parents. Sequence, Southern and RT-PCR analysis demonstrates that the CCTG expansion is located in intron 1 of the *zinc finger protein 9* (*ZNF9*) gene. The normal function of ZNF9, as a nucleic acid-binding protein, appears unrelated to any of the proteins encoded in the DM1 region of chromosome 19. Similarly, other genes in the DM2 region (*KIAA1160*, *Rab 11B*, *glycoprotein IX*, *FLJ11631*, and *FLJ12057*) bear no obvious relationship to the genes at the DM1 locus indicating that the clinical features of DM1 and DM2 are not related to disruptions in the regulation of genes in the vicinity. Similar to the CUG RNA foci in DM1, intense CCUG nuclear foci are found in DM2 muscle. Clinical and molecular parallels between DM1 and DM2 indicate that CUG and CCUG expansions in RNA can themselves be pathogenic and cause the multisystemic features common to both diseases.

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42. Clinical and genetic correlations of myotonic dystrophy type 2

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Myotonic dystrophy (DM) causes a distinctive constellation of seemingly unrelated clinical features that can be caused by mutations on either chromosome 19 (DM1) or 3 (DM2). To better define the pathophysiological mechanisms underlying DM we have studied families with DM2, and have identified a pathogenic CCTG expansion of highly variable length within intron 1 of the *zinc finger protein 9* gene (see Ranum, et al). We now report on the clinical and molecular correlations of this mutation in approximately 250 affected individuals from 82 families.

Individuals affected by DM2 have a complex clinical presentation that is strikingly similar to DM1: a characteristic pattern of weakness involving facial, neck flexor, finger flexor and hip girdle musculature; iridescent cataracts that can be symptomatic in the 3rd decade of life; hypotestosteronism and oligospermia in males; hypogammaglobulinemia, insulin insensitivity, and elevated creatine kinase; potentially fatal cardiac involvement involving severe arrhythmias or progressive cardiomyopathy. As in DM1, skeletal muscle biopsies show the characteristic features of severe fiber atrophy and a profusion of central nuclei, with intranuclear RNA inclusions in essentially all nuclei. To investigate whether splicing alterations found in DM1 are also present in DM2, we performed RT-PCR analysis of insulin receptor (IR) mRNA in skeletal muscle, which showed a marked reduction in the IR-B isoform in DM2 (18%, n=3) and DM1 (21%, n=9) compared to controls (73%, n=5). We investigated various aspects of disease severity and compared them to CCTG repeat length but found no correlation, which is partly explained by the heterogeneity of CCTG repeat length within individual samples.

In conclusion, the DM1 CTG and DM2 CCTG expansions cause a similar constellation of clinical features, similarly abnormal muscle histology with RNA inclusions, and comparable insulin receptor splicing abnormalities. The DM2 mutation is much larger and more variable in length than the DM1 mutation, with marked somatic heterogeneity and a time-dependent variation in repeat length that prevent determination of clinical severity or age of onset from measurement of the DM2 CCTG repeat length in blood.

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43. Myotonic Dystrophy: a simple mendelian disorder as model of hypercomplexity

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Although the trajectory of myotonic dystrophy (DM1) phenotype cannot be predicted on a time scale, the disease ineluctably progresses towards the expression of several clinical manifestations of aging, including those associated with both the andropause phenotype and the plurimetabolic syndrome. Aging is a misunderstood highly complex phenomenon which implies the emergence of multiple cross-interactions between environmental and genetic factors that possess various equilibria, are not controlled in a centralized manner and whose trajectory cannot be predicted. The aim of the present study was to document andropause and metabolic syndrome known covariates in DM1 and to analyse relations observed between them.

A total of 136 male DM1 subjects were assigned to a neuromuscular involvement class according to a validated scale. The association of total testosterone, FSH and LH concentrations with DM1's progression, age, disease duration and metabolic covariates, such as insulin tolerance, plasma cholesterol (C), apolipoprotein (apo) B, triglyceride (TG) HDL-C and LDL-C level was assessed, while FSH and LH response to a LHRH stimulation was evaluated.

DM1 disability correlated with testosterone ($rs = -0.31$, $P<0,001$), LH ($rs = 0.52$, $P<0,001$) and FSH ($rs = 0.54$, $P<0,001$) levels. Fasting insulin positively correlated with plasma TG, FSH and LH levels and with muscular disability ($P<0.05$), whereas the association of apo B and LDL-C concentrations with DM1 severity was not linear across their distribution and tended to reflect DM1 cell membrane damage progression. DM1 disability was not associated with an increased arterial blood pressure.

These results suggest that DM1 could be a simple mendelian trait allowing to facilitate the clinical and pharmacogenetic study of complex phenotypes and relationship between associated metabolic variables.

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44. Large CTG expansion altered the proliferative capacity of DM-1 satellite cells

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The aim of this study is to evaluate the effect of large CTG repeats on proliferative capacity of DM-1 satellite cells. *In vivo*, activation of the quiescent satellite cells leads to the generation of myoblasts, which undergo multiple rounds of division prior to fusing with existing or newly formed myofibers. The satellite cells are a population of myogenic precursor cell, which are responsible for the pre-and postnatal growth of the skeletal muscle and its regeneration. Human satellite cells were isolated from the quadriceps muscles of 3 congenital DM-1 (CDM) foetuses. Southern blot analysis showed that the 3 skeletal muscle cell cultures have similar CTG expansion, about 2300 CTG repeats. In this *in vitro* system, the proliferative life span of the CDM myoblasts isolated from 3 different patients was reduced by about 50% when compared to age-matched myoblasts isolated from unaffected individuals. Analysis of the CDM cell cultures showed that the earlier growth arrest of the CDM myoblasts results from the activation of a senescent rather than an apoptotic process. Preliminary data showed that the alteration in the proliferative capacity of CDM satellites cells is not correlated with an excessive reduction of the telomer length suggesting that the cells isolated from CDM foetuses have made more divisions *in vivo* than the aged matched non-affected satellite cells. In contrary, the CDM myoblasts stopped dividing with large telomeric DNA restriction fragments. Our data suggests that large CTG amplification interfers with the proliferative capacity of the CDM myoblasts by triggering premature senescence.

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45. Congenital myotonic dystrophy : defective satellite cells

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We have developed an *in vitro* cell culture system, which displays the majority of the defects previously described for congenital myotonic dystrophy (CDM) muscle *in vivo*. Human satellite cells were isolated from the quadriceps muscles of 3 CDM foetuses with different clinical severity. By southern blot analysis all 3 cultures were found to have approximately 2300 CTG repeats. This CTG expansion was found to progressively increase in size during the proliferative life span confirming an instability of this triplet in skeletal muscle cells. The CDM myoblasts and myotubes also showed abnormal retention of mutant RNA in nuclear foci, as well as modifications in their myogenic program. The proliferative capacity of the CDM myoblasts was reduced and a delay in fusion, differentiation and maturation was observed in the CDM cultures when compared to unaffected myoblast cultures. The clinical severity and delayed maturation observed in the CDM foetuses were closely reflected by the phenotypic modifications observed *in vitro*. Since the culture conditions were the same this suggests that the defects we have described are intrinsic to the program expressed by the myoblasts in the absence of any trophic factors. Altogether, our results demonstrate that satellite cells are defective in CDM and are probably implicated in the delay in maturation and muscle atrophy that has been described previously in CDM foetuses.

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46. Increase activation of subcortical structures in myotonic dystrophy: a functional MRI study

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Pathologic and imaging studies have documented an involvement of central nervous system in myotonic dystrophy (DM1) including white matter lesions, atrophy and alterations in the basal ganglia and thalamus. Functional MRI (fMRI) may reveal compensatory patterns of activation in the presence of cerebral lesions, which do not affect execution of a task. We investigated the patterns of cerebral activation in patients with DM1 during a simple motor task. Thirteen right handed DM1 patients (8 men and 5 women, aged between 19 and 55 years, median 29 years), with disease severity ranging from 1 to 4 (average score 2.8±0.7) [*Mathieu 1992*] and 10 matched healthy controls were scanned on a 1.5 Tesla during a sequential finger-to-thumb opposition task of right- and left-hand, both separately and together. Group analysis of both patients and controls showed a similar extent of activation in the contralateral sensorimotor cortex and ipsilateral cerebellum during both right- and left-hand movement, and in the ipsilateral motor cortex during left hand movement. The bilateral task was associated with bilateral activation of the sensorimotor cortex and cerebellum. Interestingly, a significant activation of bilateral basal ganglia was observed during all three motor tasks in the DM1 group compared to controls ($p<0.05$). An increased activation of basal ganglia during a simple motor task may be related to a subclinical involvement of these structures in DM1. Alternatively it may represent a central facilitatory response to peripheral motor impairment.

Mathieu J, De Braekeleer M, Prost C, Boily C. Myotonic dystrophy. Clinical assessment of muscular disability in an isolated population with presumed homogeneous mutation. Neurology 1992; 42:203-208.

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47. Dysregulation of human brain microtubule-associated tau mRNA maturation in myotonic dystrophy type 1

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Intraneuronal aggregates of hyperphosphorylated tau proteins, referred to as pathological tau, are found in brain areas of demented patients affected by numerous different neurodegenerative disorders, as Alzheimer's disease, Pick's disease, progressive supranuclear palsy, fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). The biochemical analysis of pathological tau enables to distinguish 4 classes of tau pathology. The fourth corresponds to the biochemical profile of pathological tau protein in myotonic dystrophy type 1 (DM1), characterized by a single major component of 60 kDa. In the human central nervous system, tau proteins consist of six isoforms that differ by the presence or absence of the alternatively spliced exons 2, 3 and 10. Tau isoform expression was investigated in five patients with DM1. Here we show that the pattern of tau isoforms aggregated in DM1 brain lesions is characteristic. It consists mainly of the aggregation of the shortest human tau isoform. A disruption in normal tau isoform expression was demonstrated both at the mRNA and protein level consisting of a reduced expression of tau isoforms containing the exon 2. DM1 is characterized by an unstable CTG repeat expansion in the 3' untranslated region of the DM-protein kinase gene. Among recent hypotheses of the aetiology of DM1, trans dominant effect of CUG expansion upon the post-transcriptional maturation of gene products have been proposed. For that purpose, tau protein expression was investigated in transgenic mice with large human genomic DNA including the DMPK gene with the normal or the expanded allele as well as in DMPK knockout mouse. Mouse brain tau protein expression was also altered in homozygous mice with DM1 mutation whereas it remained normal in the other transgenic mice as well as in DMPK knock out mice. Histological analysis of brain tissue of transgenic mice failed to show any changes in brain cytoarchitecture as well as any tau protein inclusions. Together, our data suggest a relationship between the CTG repeat expansion and the alteration of tau expression showing that DM1 is a peculiar tauopathy. Moreover, our results demonstrate that any change in tau protein expression, as observed in FTDP-17, results in neurodegeneration and DM1 is the first neurological disorder linked to the change of tau exon 2 inclusion.

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48. Abnormal cardiac magnetic resonance imaging findings in myotonic dystrophy with inducible ventricular tachyarrhythmias

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Purpose: To prospectively determine in patients with myotonic dystrophy (MD) whether a correlation exists between morphological and functional abnormalities of the right ventricle by magnetic resonance (MR) imaging and electrophysiologic (EP) testing findings.
Methods and Results: Electrocardiographically-gated T1-weighted conventional and black blood single-shot fast spin-echo and cine MR sequences were performed in 30 MD patients who required EP testing. Patients were divided in 2 groups according to the results of EP testing: inducible = patients with inducible ventricular tachyarrhythmias during EP studies (n=10), versus non inducible = patients without inducible ventricular tachyarrhythmias during EP studies (n=20).
Results: Increased signal-intensity of the right ventricular wall indicative of fatty replacement, was identified by 2 independent investigators in 7 inducible patients, along with contraction abnormalities in 5 patients and myocardial thinning of the right ventricle in 5 patients, including 1 patient without fatty infiltration. No hyperintensity, myocardial thinning or contraction abnormalities of the right ventricular wall were observed in non inducible patients.
Conclusions: This study suggests the existence of a relationship between inducible ventricular arrhythmias and MR morphologic and functional abnormalities of the right ventricle in MD patients. The confirmation of such correlation may legitimate the use of MRI imaging as a non invasive method to estimate the individual risk of ventricular arrhythmias in MD patients.

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49. Correlation between cardiac conduction disease and CTG expansion size

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Cardiac involvement, in the form of conduction block and arrhythmia, is common in myotonic dystrophy (MyD). It is a cause of both morbidity and mortality, and it has been presumed, but is unproven, that those occasional cases of sudden, "unexpected", death in MyD relate to cardiac disease. In general terms, the phenotypic expression of MyD correlates with the size of the CTG expansion; larger expansions are associated with earlier onset and more severe disease. Previous cross-sectional studies have suggested that this generalization is also true for cardiac involvement. Despite this, there remain many uncertainties concerning the prediction of cardiac events, and thus uncertainty as to the practical advice that should be offered to such patients concerning cardiac screening. The present study is, we believe, the first longitudinal study relating CTG expansion size to progression of cardiac disease.

Seventy three patients attending the Oxford regional muscle clinic underwent longitudinal ECG and clinical follow-up over a mean of 4.8 and 6.2 years respectively. They were stratified on the basis of CTG expansion in lymphocytes: (E0/1 n=25; E2 n=34, E3/4 n=14). The proportion of patients with a QRS complex >100ms at baseline increased with the size of the CTG expansion: E0/1 4%, E2 12%, E3/4 36%, p=0.02. This trend was more pronounced at follow-up: E0/1 4%, E2 21%, E3/4 57%, p=0.0004. The rate of widening of the QRS complex (ms/yr) also related to the size of the expansion: E0/1 0.4, E2 1.4, E3/4 1.5, p=0.04. First-degree atrioventricular block was present in 23% at baseline and 34% at follow-up, but did not show a significant relation to expansion size. Seven patients suffered a cardiac event during follow-up [sudden death (2), pacemaker insertion (3), chronic atrial arrhythmia (2)] of whom six were expansion size E2 or greater. Patients who experienced a cardiac event had more rapid rates of PR interval increase (9.9 *v* 1.6 ms/yr).

In conclusion, larger CTG expansions are associated with a higher rate of conduction disease and an increased risk of cardiac events. A normal ECG predicts a low risk of cardiac events in the medium term. An annual ECG and symptomatic assessment is adequate for most patients, in whom the progression of cardiac disease is slow.

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50. Contractile activity of cardiac myocytes isolated from *Dmpk* KO mice

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Dysfunction of the gene encoding *DMPK* (myotonic dystrophy protein kinase) has been implicated in the human neuromuscular disease myotonic dystrophy (DM1). The cardiac features of the disease include progressive development of conduction defects, which often underlie the cause of "sudden death" in DM1 patients. These cardiac conduction defects have been observed in whole hearts of mice deficient for *Dmpk* function (Berul *et al.*, J.Clin.Inv.1999; 103:R1-7).

We have used single cell physiology and protein biochemistry to investigate contractile activity of cardiac myocytes isolated from *Dmpk* KO mice. *DMPK* encodes a serine/threonine kinase related to the Rho protein kinase family involved in cytoskeletal organisation and calcium (Ca^{2+}) sensitisation of contractility. Contraction of cardiac cells is activated by the release of Ca^{2+} stored in the sarcoplasmic reticulum (SR). The Ca^{2+} is cycled back to the SR to achieve relaxation of the muscle. Re-entry of Ca^{2+}into the SR is determined by the activity of the cardiac SR Ca^{2+}ATPase which is regulated by a phosphoprotein phospholamban (PLB).

We demonstrate a deficit in *Dmpk* leads to enhanced basal cardiac myocyte contractility. Results indicate altered Ca^{2+}uptake into the SR via changes in PLB phosphorylation. Concurrent with hyperphosphorylated PLB the contractile response to β–adrenoreceptor agonist isoprenaline was reduced.

These observations suggest a modulatory role for DMPK in SR function and implicate SR Ca^{2+} dysregulation in the development of cardiac complications in DM1 patients.

WIP

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51. Genotype-phenotype correlation of otolaryngologic findings in myotonic dystrophy type 1

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Background: Otolaryngologic examinations that include eye movement and stapedial reflex (SR) tests can contribute to the quantitative evaluation of muscular involvement in DM1.
Object and Methods: We studied oculomotor functions in 29 DM1 patients and in 11 age-matched controls. We also report a case-control study that compared the SR waveform (latency: L, contraction time: C50, and relaxation time: D50) measured by the acoustic impedance method in 13 patients with DM1 and in 14 control subjects matched for age and sex. The correlation between these findings and CTG repeat ((CTG) n) length in DM1 patients obtained by Southern blot analysis with Eco R I was also examined. MRI findings of the extraocular muscles and swallowing functions will also be mentioned briefly.
Results: 1. Oculomotor functions. Values for saccadic velocity (P<.005), maximum slow phase velocity of the optokinetic nystagmus (OKNspv) (P<.005), and maximum slow phase velocity of the caloric nystagmus (CNspv) (P<.01) in the patient group were markedly
lower than in the control group. Visual suppression of caloric nystagmus (VS) in the patient group was slightly lower than that in the controls. No significant difference was found between the two groups in the maximum frequencies of optokinetic nystagmus (OKNfq). Patients with greater (CTG)n lengths had lower saccadic velocities (P<.01, r=.71). 2. Stapedial reflex. We found (1) no significant difference between the pure tone audiometric threshold at 500 Hz in the DM1 patients and that in the control subjects; (2) nor between the SR thresholds in the patients and controls (500 Hz stimuli); (3) C50 and D50 in DM1 patients to be significantly prolonged, whereas L was not; (4) C50 and D50 in DM1 patients to be significantly correlated with CTG repeat length, whereas L was not.
Conclusion: Our findings show that eye movement and SR tests can be used for a quantitative evaluation of muscle involvement in DM1. We consider that the diminished saccadic velocity and prolonged C50 are caused by weakness of extraocular muscles or stapedial muscle, and prolonged D50 by myotonia of stapedial muscle.

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52. High resolution haplotype analysis in the DMWD-DMPK-SIX5 region

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To understand the mechanism of the ancient mutation or evolution, population genetics is important. Although several models have been proposed, mechanism of DM1 ancient mutation remains uncertain. We previously reported that Japanese DM1 alleles were always haplotype A, the same as Caucasians, and proposed the multistep model of CTG repeat expansion. Here, we report high resolution haplotype analysis using over 300 DM1 Japanese family members. We examined more than 10 SNPs at the 26kb DMWD-DMPK-SIX5 region using PCR-RFLP methods, TaqMan-PCR or allele-specific PCR methods. Novel polymorphisms we examined are as follows: microsatellite at the upstream region and SNP in intron 1 (DMWD gene), SNPs in promoter region (DMPK), SNPs between 3'-UTR of DMPK and promoter region of SIX5, SNPs in exon 3 (SIX5). As expected, strong linkage disequilibrium was observed when normal alleles were grouped according to CTG repeat length. However, the situation was different between promoter of DMPK and exon 3 of SIX5. In the former case, 5 and more than 20 CTG repeat including DM1 were always linked to G allele. In the latter case (18kb centromeric), more than 20 CTG repeat were only linked to C (Leu) allele. This suggests that a major ancient mutation underlying DM1 has originated by reservoir pool (CTG19-37), not by CTG5. Detailed haplotype analyses are in progress.

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53. Myotonic dystrophy : frequency and characteristics of diagnosis missing by physicians in France

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Introduction : Myotonic dystrophy (DM) is one of the most frequent dystrophies in adult. Diagnosis may be unknown for a long time, although it would be performed easily on familial history and characteristic clinical manifestations.
Aim of study : to appreciate the frequency and the characteristics of DM diagnosis missing by physicans in the French population.
Material and methods : 85 DM patients from Institute of Myology database were studied (44 males, 41 females, mean age : 41.6 years). Diagnosis was considered as "missed" by the physician (generalist or specialist) when the two following conditions were simultaneously present : 1) medical advice required by a DM patient for a) a characteristic symptom of the disease (see above), or b) the systematical survey of a pregnancy, or c) unexplained neonatal death / late onset abortion; 2) DM diagnosis available at the moment of consultation : a) presence of characteristic signs or symptoms (myotonia, early onset cataract, distal muscle wasting, neonatal or infantile DM, and/or b) typical familial history of DM well-known by the patient.
Results : Diagnosis was missed by the physician in 51 out of 85 cases (60%). The mean age at "missed consultation" was 25.3 years (3 to 60 y). DM symptoms and/or signs leading to the consultation, but not to the diagnosis of the disease were the followings (number of cases) : myotonia (21), cataract (11), arrhythmia and/or conduction defect (8), weakness (4), neonatal DM baby born to symptomatic mother (8), late onset abortions (2), significant school retardation (3), marked speech difficulties (4), infertility (4), face/jaw abnormality (2), significant gastrointestinal tract problems (1).The time elapsed between the first symptom and DM diagnosis was 13.5 +/- 9.6 years.. The mean delay between effective diagnosis and the "missed" consultation was 9 +/- 7.3 years for myotonia, 8.8 +/- 7.26 y for cataract, 6.75 +/- 6.1 y for cardiac problems. The categories of physicians missing diagnosis were the followings : general physician (21), ophthalmologist (11), pediatrician (9), cardiologist (8), gynecologist-obstetrician (7), gastroenterologist (4), oto-rhino-laryngologist (4), neurologist (2), urologist (2), maxillo-facial surgeon/stomatologist. (2).
Conclusions. Even in a high medical level country as France, DM is poorly known by physicians and DM diagnosis is very often missed. A better education is necessary to diagnose early the disease and detect the potentially severe heart risk and warrant a proper genetic council (neonatal DM prevention).

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54. Outcomes of presymptomatic testing for myotonic dystrophy

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Analysis of 8 years' experience of presymptomatic testing for myotonic dystrophy in Wales has shown 78 presymptomatic tests over this period, 72 involving staff of the clinical genetics service, with full details available. No tests were done on healthy children. 16 individuals showed an abnormal result (20.5%), but 9 of these showed some clinical abnormality on examination, despite considering themselves healthy, so the chance of a clinically normal individual receiving an abnormal result was only 10.1%. Only 2 clinically normal individuals showed an expansion exceeding 100 repeats, giving a risk of less than 3% of a clinically normal person being found to have a clinically significant abnormal result.

These results are of relevance to genetic counselling related to presymptomatic testing and confirm the high penetrance of the myotonic dystrophy mutation in established families.

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55. Type 2 fiber atrophy in *DM2*-linked PROMM - diagnostic distinction to myotonic dystrophy (DM1)

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Myotonic dystrophy-type 2 is a dominant multiorgan disease with similarities to myotonic dystrophy-type 1 (DM1, Steinert's disease). The clinical course is usually milder; there is no prominent distal or facial muscle atrophy, no congenital form with cerebral symptoms has been determined and there are no severe respiratory complications. (CTG)n expansion mutation on chromosome 19q as with DM1 is lacking, whereas the cataracts are DM1-like. Clinical myotonia is variably present, myalgia can be prominent, symptoms may be aggravated by hormonal influence, and cardiac disturbances may occur. Proximal muscle weakness is a core feature but clinical atrophy is infrequent. Because of the large clinical variability, different families have been described as proximal myotonic myopathy (PROMM), proximal myotonic dystrophy (PDM) and myotonic dystrophy type 2 (DM2). Most PROMM families as well as the PDM and DM2 families show linkage to the *DM2*-locus on chromosome 3q. On muscle biopsy the large number of internal nuclei, pyknotic nuclear clumps, and very rare necrotic or regenerative fibers are similar findings as with DM1. Sarcoplasmic masses, fiber type 1 atrophy and frequent ringed fibers, however, are usually not present in *DM2*-linked PROMM, whereas and fiber type 1 predominance has been recognized. In this study we present selective fiber type 2 atrophy in *DM2*-linked PROMM as a new finding which can be used for clinical differential diagnosis, and for confirmation of diagnosis in sporadic patients. Fiber type 2 atrophy is more easily detected with immunocytochemistry for fiber type differentiation than with conventional ATPase reaction because many of the atrophic fibers are extremely small.

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56. Prevalence of excessive daytime sleepiness in myotonic dystrophy

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Excessive daytime sleepiness is a frequent and disabling symptom in myotonic dystrophy. The Epworth sleepiness scale (ESS) and the multiple sleep latency test (MSLT) are used commonly to assess the daytime sleepiness. 87 patients with myotonic dystrophy were prospectively subjected to an ESS. When excessive daytime sleepiness was presumed higher than 10 on ESS, patients underwent a polysomnography with MSLT. Patients with a MSLT lower or equal to 8 minutes were considered to have proven daytime sleepiness. By using ESS, 40 patients had excessive sleepiness with a mean of 14,8+/-2,9, among them, 15 patients had a MSLT lower or equal to 8 min. Prevalence of excessive daytime sleepiness in myotonic dystrophy is about 50% with ESS and 17 % with MSLT.

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57. The electromyographic study of dehydroepiandrosterone sulfate in patients with myotonic dystrophy

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Objectives: To evaluate the effect of dehydroepiandrosterone sulfate (DHEAS) therapy in myotonic dystrophy (DM) by electromyographic (EMG) analysis.
Background: We reported that DHEAS therapy is effective to reduce DM patient's myotonia. However, it is not evaluate the EMG changes of intravenous administration (IV) of DHEAS preparation in DM patients.
Design/Methods: The IV of a DHEAS 200 mg/day every day for 8 weeks or twice a week for 2 to 10 months were performed to 13 DM patients (male 5 and female 8). Full informed consents were obtained from all patients. Evaluation made by myotonia grade and EMG examination before and the end of DHEAS therapy. The severity of myotonia was assigned a grade 0 to 4 by a clinical evaluator. The surface EMG was examined at thenar muscles by repetitive stimulation (10Hz, 50 times) to median nerve. The duration of after-discharge (AD) were measured from the end of repetitive stimulation to the disappearing point of AD. The minimum numbers of times at the appearance of AD were also examined.
Results:(1) Myotonia Grade: Remarkable improvement 6 patients (disappearance 4) and moderate in 2 patients were observed in 13 patients at the end of DHEAS therapy ($p<0.01$). (2) EMG findings: The duration of AD were shortened in 11 patients ($p<0.01$), and the minimum number of times of the appearance of AD were also prolonged in 7 patients ($p<0.05$). (3) In each patient, change of severity of myotonia was reflected by those of duration and minimum number of time of AD in EMG findings.
Conclusions: Our findings suggested that DHEAS therapy is effective for myotonia in patients with DM, and that is supported by EMG findings. Our method of EMG examination is insufficient to compare each cases directly, but I think it is suitable method to observe the changes in an individual case through DHEAS treatment in DM patients.

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58. The effect of dehydroepiandrosterone sulfate on myotonia: Intracellular study

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In myotonic dystrophy dehydroepiandrosterone is reduced in the serum, and Sugino and others reported that dehydroepiandrosterone sulfate (DHEAS) has therapeutic effects for the patients by reducing myotonia and cardiac arrhythmia, and improving daily activities of living. In order to study the
effects of DHEAS on myotonia, we have performed intracellular recordings on the hemidiaphragm preparations obtained from mto mice (myotonic mice, SWR/J-Clcn1adr-mto), which were imported from Jackson Laboratory, U.S.A. Isometric twitch tension was studied by using the phrenic hemidiaphragm preparations obtained from Wistar rats to investigate the effects of DHEAS and mexiletine on the muscle tension. The results indicated that the mean resting membrane potentials (RMP) of mto hemidiaphragm prepations was –79.7±5.5 (SD) mv, and the glass microelectrode insertion elicited myotonic bursts in almost all the muscle cells in Tyrode solution. When DHEAS was added to Tyrode solution at various concentrations, the percentage of insertion myotonia reduced to 55% of the impaled muscle fibers and the mean RMP was –79.9±4.8 mv at the concentration of 80mg/L, and insertion myotonia further reduced to 30% and the mean RMP was –83.1±5.3 mv at the concentration of 160mg/L, and no insertion myotonia was noted and the mean RMP was hyperpolarized to –89.8±5.6 mv at the concentration of 320mg/L. In the phrenic hemidiaphragm preparations of Wistar rats DHEAS reduced isometric twitch tension only up to 70% of the original muscle tension even when DHEAS was added to Tyrode solution at 320mg/L. Mexiletine which has been used for myotonia reduced isometric twitch tension to 30% of the original muscle tension at the concentration of 10mg/L. For myotonic dystrophy it is helpful to have such a drug as DHEAS to reduce myotonia and yet does not reduce muscle power, since progressive muscle weakness occurs in myotonic dystrophy.

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Comments

Suppressive effect of dehydroepiandrosterone sulfate on myotonia by interacting with Na^+ channel

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Objective: To uncover the molecular basis of the suppressive effect of dehydroepiandrosterone sulfate (DHEAS) on myotonia.
Background: Myotonic dystrophy is a type of autosomal-dominant muscular dystrophy, associated with a number of systemic symptoms in addition to myotonia. Recently, dehydroepiandrosterone sulfate (DHEAS) was tried on 11 patients with myotonic dystrophy giving favorable results, as DHEAS decreased myotonia and improved their activities of daily living, but the mechanism of DHEAS action for myotonia was unknown.
Methods: The effect of DHEAS was studied on the recombinant skeletal muscle Na^+ channel expressed in HEK cells.
Results: Whole-cell mode of patch clamp studies on the recombinant Na^+ channel showed that DHEAS reduced Na^+ channel currents immediately on application, and that the effect was quickly reversible upon wash out. The decrease in Na^+ current was not only dose-dependent, but also strongly dependent on the membrane potential. Although DHEAS shifted the voltage-dependence of inactivation obtained using the conventional prepulse protocol to the negative direction, the recovery time from the inactivated state was not prolonged, nor use-dependent block was observed in the presence of DHEAS.
Conclusions: DHEAS preferentially interacts with the Na^+ channel in the depolarized state to suppress myotonic discharges, most likely blocking the channel pore from the extracellular side.

59. Molecular mechanisms of dehydroepiandrosterone-sulfate effects on skeletal muscle cells

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It has been reported that intravenous administration of dehydroepiandrosterone sulfate (DHEA-S) was effective for the major symptoms of myotonic dystrophy type 1 (DM1), and a trial with oral administration of DHEA-S showed possible efficacy for neuropsychological/cognitive symptoms in DM1 patients. However, no receptors for DHEA(-S) has been identified and molecular mechanism(s) of their actions remain unknown. As a step to clarify the molecular mechanism(s) of DHEA and DHEA-S actions, we studied the intracellular transport of DHEA-S and the activation of nuclear receptors: (i) we showed that the CD36/FAT could transport DHEA-S from extracellular space to cytoplasm in adult human concentration of DHEA-S. Expression level of CD36/FAT in skeletal muscle cells increased developmentally, as DHEA-S transport ability increased in parallel, suggesting the actual transport of DHEA-S into matured skeletal muscle cells *in vivo*. After transport into intracellular space, DHEA-S may act via association with cytosol or nuclear components. The fact that DHEA-S binding sites in skeletal muscle are established might support the direct actions of DHEA-S via such mechanism, (ii) electrophoretic mobility shift assays revealed that *in vivo* treatment of DHEA-S, more effectively than of DHEA, could activated CARβ/RXR nuclear receptors, and increased nuclear RXR protein in mouse liver. DHEA and DHEA-S could be metabolized *in vivo* and act as ligands for nuclear receptors, although DHEA and DHEA-S can not activate CARβ-RXR directly. These findings suggest that molecular mechanisms of DHEA/DHEA-S actions would be diverse, other than previously reported rapid modulation of ion channel function. Now we are investigating effects of CD36/FAT or nuclear receptors on the development and the function of skeletal muscle cells.

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60. Developing a tissue culture model of CTG instability using DM1 patient cells

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Work in progress

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61. Chemotherapeutically induced deletion of expanded triplet repeats

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Genetic assays were developed to measure rates of deletion of one or more (CAG)•(CTG) repeats, or an entire repeat tract, in *Escherichia coli*. In-frame insertions of ≥_25 repeats in the chloramphenicol acetyltransferase (CAT) gene of pBR325 resulted in a chloramphenicol sensitive (Cm^s) phenotype. Deletion of one or more repeats resulted in a chloramphenicol resistant (Cm^r) phenotype. When $(CAG)_{25}$ comprised the leading template strand, mutation to a Cm^r phenotype occurred at a very high rate of $1 \cdot 10^{-3}$ mutations per plasmid per replication. The mutation rates for plasmids containing $(CAG)_{43}$ or $(CAG)_{79}$ decreased significantly. In a second assay, out-of-frame mutation inserts underwent complete deletion at rates ranging from about $5 \cdot 10^{-9}$ to $1 \cdot 10^{-7}$ mutations per cell per generation. These assays allow careful quantitation of triplet repeat instability in *E. coli* and analysis of the effects of mutations in genes involved in replication, repair, and recombination. Applying these sensitive genetic assays in *E. coli*, we also demonstrate that certain DNA damaging agents, including anticancer agents, increase the rate of triplet repeat deletion in a length dependent fashion. In addition, human lymphoblast DM1 cell lines containing expanded triplet repeats have been treated with some of these agents. Following treatment and growth, in general, the continued expansion observed in DM1 cells in culture appears to be prevented and in many cases large deletions in the repeats are observed. These results suggest that a chemotherapeutic approach to the reduction in triplet repeat length may provide one possible rationale to slow, stop, or reverse the progression of CTG-associated diseases. This may be particularly useful if agents that selectively interact with the repeat tract could be identified.

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62. The use of RNA antisense as a potential gene therapy for myotonic dystrophy (DM)

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The complex and variable phenotype observed in DM is most likely caused by a complex molecular pathogenesis, including deficiency of DMPK protein, a trans-dominant misregulation of RNA homeostasis and haplo-insufficiency of a neighboring homeobox gene (particular the DM locus-associated homeodomain protein (DMAHP gene). Gene therapies directed toward dominant diseases, where the pathology is due at least in part to a gain of function mutation rather than haplo-insufficiency alone, may require suppression of the disease allele while maintaining expression of the wild-type allele. As a first approach to develop a specific treatment for DM, we first develop an antisense RNA gene therapy to target DMPK mRNAs. Using antisense oligonucleotides we determined that the CUG expansion could be a potential RNA antisense target in the DMPK mRNAs. We then construct a retrovirus expressing both a (CUG)5 antisense RNA and the GFP protein. DM myoblasts were infected with recombinant retrovirus and GFP-positive cells were purified by cell sorting. By northern blot, we showed that the transgene were expressed at high levels. The levels of the mutant DMPK transcripts were decreased by more than 80% whereas the levels of the mutants transcripts were decreased by 40-50%. These cells proliferated at a similar rate as uninfected or parental cells. We previoulsy published that the insulin resistance was retained in differentiated DM myoblasts. Treatment of uninfected DM myotubes with physiological dose of insulin (10 nM) did not increase the uptake of glucose whereas treatment of infected cells with 10 nM insulin increased by 2-fold the uptake of glucose. Finally, the expression of antisense completely inhibited CUG-binding activity of several CUG-binding proteins including CUGBP1. These data suggest that antisense RNA could be a potential gene therapy for DM.

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63. A novel RNA targeting approach for the repair of the myotonic dystrophy defect

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Ribozymes are RNA molecules, which possess catalytic activity. Several ribozyme classes have been investigated for their potential to modify genetic information. Such properties are important for the development of therapeutic strategies against many diseases. One of these types of ribozymes, the group I intron, is capable of targeted RNA trans-splicing. The Tetrahymena group I intron is an RNA molecule of approximately 400 bases which catalyses its own intron splicing. The reaction proceeds by two consecutive transesterification reactions in the presence of a divalent cation. Recently, it has been shown that the group I intron ribozyme can replace part of a target RNA molecule with a sequence, contained at the ribozyme's 3' end. A uridine preceding the target cleavage site is the only sequence requirement. Group I intron ribozymes have been used successfully to repair mutant transcripts in cell culture.

The genetic defect of myotonic dystrophy (DM) can be repaired using the group I intron *trans*-splicing properties. An amplified trinucleotide repeat expansion (TRE) in the 3' untranslated region of the myotonic dystrophy protein kinase (DMPK) gene is the cause for DM. The molecular pathogenesis of DM is quite complex and is thought that the TRE mutation is responsible for many molecular abnormalities leading to DM. The mutant DMPK mRNA seems to be playing a critical role in the pathogenesis of the disease since it is trapped in the nucleus of the affected cells.

Our approach aims to take advantage of the group I intron ribozyme's *trans*-splicing properties to repair the genetic defect which gives rise to DM. A group I intron ribozyme has been used to *trans*-splice the DMPK transcirpt in cells. It has been shown that it can *trans*-splice part of the DMPK RNA with the wild type sequence, engineered to be carried in the ribozyme. The reaction has been performed in high fidelity and a base-specific manner. *Trans*-splicing products have been also detected when a ribozyme construct has been used to transfect human fibroblasts expressing the DMPK gene.

Ribozyme-mediated repair is a promising approach for the therapy of DM. There are parameters, however, which should be investigated in order to make this method more efficient and reliable such as the ribozyme delivery, specificity and efficiency of *trans*-splicing.

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64. Excessive daytime sleepiness in myotonic dystrophy

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Excessive daytime sleepiness, or hypersomnolence, is a common but under-recorded, symptom of myotonic dystrophy. Rarely, it may be due to hypoventilation secondary to respiratory muscle weakness, with or without additional obstructive sleep apnoea – in such cases the problem is alleviated by respiratory support (e.g. nocturnal ventilatory support). More commonly it has a central, and as yet unexplained, aetiology. A handful of previous reports have reported benefit from traditional stimulant drugs such as dexamphetamine and methylphenidate, but for obvious reasons these have not been widely used.

The current study is divided into two areas: a) A survey of a specific clinic population aimed at determining the prevalence and severity of the problem, and b) A double-blind crossover study of the newly available stimulant drug modafanil.

Survey

The Epworth Sleepiness Scale (ESS) is a widely used, and well-validated, rating scale of sleepiness. A score of 10 or above is considered abnormal, a score of greater than 15 reflecting a high degree of daytime somnolence. Patients with myotonic dystrophy attending the Oxford muscle clinic are being invited to complete an ESS questionnaire. The most recent analysis was of 69 patients. Fifty (72%) had an ESS of ≥ 10, and 26 (38%) ≥ 15. These figures are comparable to those seen in narcolepsy and obstructive sleep apnoea, and indicate considerable morbidity. There was no correlation with expansion size.

Modafanil

We have commenced a double-blind crossover study of modafanil and anticipate recruiting 20-30 patients. Polysomnography and various tests of sleepiness (MSLT, etc) and alertness (driving simulation) are being used to assess the effectiveness, or otherwise, of the drug. The design of the trial and preliminary results will be discussed.

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65. A conceptual framework to implement a comprehensive systemic health care program in myotonic dystrophy

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Myotonic dystrophy type 1 (DM) is characterized by abnormalities in various systems leading to multiple impairments and disabilities and thus requires complex and global clinical management. Beyond the biological and clinical components, social dimensions of DM and their impacts on quality of life and well-being of affected individuals must also be documented to ensure optimal management of this disease and to implement a preventive systemic health care program.
To document phenomena such quality of life and social participation and their determinants, there is a need to use a comprehensive framework that includes all dimensions of interest. We propose to use the Handicap Creation Process (HCP) model, developed by the International Network on Handicap Creation Process of the World Health Organization (WHO). Measurement scales of social integration, specific to this model, have been constructed and validated in the Quebec population (Canada). The HCP model promotes an integrative approach (clinical-social-biological); adapted to DM, it will be useful to assess the relationships between handicap situations, quality of life, physical or mental disabilities, clinical, physiological, metabolic, genetic and environmental factors in order to identify potential determinants of well-being and social participation.
The dimensions of the HCP model will be assessed in the Quebec DM population. In order to ensure transfer of the results to other populations, the measurement scales of social integration will also be used in France, a culturally distinct population. The project will improve our understanding of the natural history of DM, will promote the emergence of comprehensive disease management protocols and will identify useful outcomes measures for future therapeutic trials.

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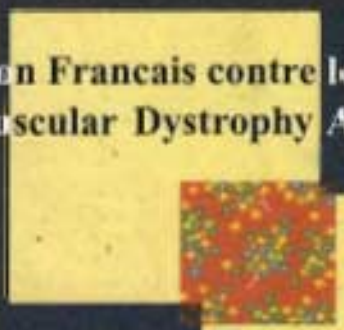

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