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GENETIC ANALYSIS OF THE X CHROMOSOME

Studies of Duchenne Muscular Dystrophy and Related Disorders

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Edited by

Henry F. Epstein

The Jerry Lewis Neuromuscular Disease Research Center
Baylor College of Medicine
Houston, Texas

and

Stewart Wolf

Totts Gap Medical Research Laboratories
Bangor, Pennsylvania
Temple University
Philadelphia, Pennsylvania

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PREFACE

The present volume contains the edited transcript of a colloquium sponsored by the Muscular Dystrophy Association and held at Mountain Shadows Inn, Scottsdale, Arizona, December 14-16, 1981.

The participants, geneticists, molecular biologists, biochemists and clinicians, explored in open dialogue ways and means of identifying and characterizing the genetic alterations responsible for X-linked muscular dystrophies, especially the Duchenne type. The clinicians, who urged the use of properly diagnosed and documented case material for study, emphasized the troublesome fact that the primary phenotypic expression of the gene (or genes) involved in the muscular dystrophies is yet to be identified.

Discussions centered on the applicability of recent methodological advances in DNA chemistry and molecular biology, cytogenetics and cell biology to mapping the X chromosome. Despite ignorance of the basic disorder in the muscular dystrophies, DNA technologies and chromosome mapping strategies for the discovery of genetic defects and phenotypic expressions were proposed. Beyond its stimulating intellectual exchange, the colloquium yielded important benefits. The participants agreed to share needed cell lines and endonuclease restriction enzymes and to organize interlaboratory communication and collaborative efforts to accelerate progress in the quest for the genetic lesion in Duchenne muscular dystrophy.

The discussions were recorded, transcribed, edited and to some extent, rearranged to fit into a sequence of chapters. The editors are grateful to Joy Colarusso Lowe whose unusual skill, patience and persistence made it possible to convert a highly specialized technical discussion into a coherent manuscript.

PARTICIPANTS

Samuel H. Boyer, IV, M.D., Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland.

Michael H. Brooke, M.D., Department of Neurology, Washington University School of Medicine, St. Louis, Missouri.

Gail A.P. Bruns, M.D., Department of Pediatrics, Children's Hospital Medical Center, Harvard Medical School, Boston, Massachusetts.

C. Thomas Caskey, M.D., Departments of Medicine and Biochemistry, Baylor College of Medicine, Howard Hughes Medical Institute, Houston, Texas.

A. Craig Chinault, Ph.D., Departments of Medicine and Biochemistry, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas.

P. Michael Conneally, Ph.D., Department of Medical Genetics, Indiana University School of Medicine, Indianapolis, Indiana.

Norman Davidson, Ph.D., Department of Chemistry, California Institute of Technology, Pasadena, California.

Richard Doherty, Ph.D., Departments of Pediatrics, Obstetrics and Gynecology, University of Rochester School of Medicine, Rochester, New York.

Charles P. Emerson, Jr., Ph.D., Department of Biology, University of Virginia, Charlottesville, Virginia.

Henry F. Epstein, M.D., Department of Neurology, Baylor College of Medicine, Houston, Texas.

Uta Francke, M.D., Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut.

James F. Gusella, Ph.D., Genetics Unit, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts.

Stephen D. Hauschka, Ph.D., Department of Biochemistry, University of Washington, Seattle, Washington.

Robert E. Hausman, Ph.D., Biological Science Center, Boston University, Boston, Massachusetts.

Barbara K. Hecht, Ph.D., Genetic Center, Southwest Biomedical Research Institute, Tempe, Arizona.

Frederick Hecht, M.D., Genetic Center, Southwest Biomedical Research Institute, Tempe, Arizona.

David Housman, Ph.D., Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts.

Oliver W. Jones, M.D., Department of Medicine, University of California, San Diego, La Jolla, California.

Simona Kahane, Ph.D., Sloan-Kettering Institute for Cancer Research, New York, New York.

Laurence H. Kedes, M.D., Veterans Administration Hospital, Palo Alto, California.

Louis M. Kunkel, Ph.D., Division of Genetics, Children's Hospital Medical Center, Harvard Medical School, Boston, Massachusetts.

Samuel A. Latt, M.D., Ph.D., Department of Pediatrics, Children's Hospital Medical Center, Harvard Medical School, Boston, Massachusetts.

Anthony N. Martonosi, M.D., Department of Biochemistry, State University of New York, Syracuse, New York.

M.L. Moss, Ph.D., Director of Research Development, Muscular Dystrophy Association, New York, New York.

Bernardo Nadal-Ginard, M.D., Ph.D., Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York.

Robert L. Nussbaum, M.D., Department of Medicine, Howard Hughes Medical Institute, Baylor College of Medicine, Houston Texas.

Bruce M. Paterson, M.D., National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

Everett C. Schreiber, Jr., Ph.D., Research Department, Muscular Dystrophy Association, New York, New York.

Robert J. Schwartz, Ph.D., Department of Cell Biology, Baylor College of Medicine, Texas Medical Center, Houston, Texas.

Lawrence J. Shapiro, M.D., Division of Medical Genetics, Harbor General Hospital, Torrance, California.

Marcello Siniscalco, M.D., Somatic Cell Genetics, Sloan-Kettering Institute for Cancer Research, New York, New York.

Kirby D. Smith, Ph.D., Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland.

Richard C. Strohman, Ph.D., Department of Zoology, University of California, Berkeley, Berkeley, California.

Paul Szabo, Ph.D., Somatic Cell Genetics, Sloan-Kettering Institute for Cancer Research, New York, New York.

Ray White, M.D., University of Utah School of Medicine, Howard Hughes Medical Institute, Salt Lake City, Utah.

Robert Williamson, M.D., Ph.D., Department of Biochemistry, St. Mary's Hospital Medical School, University of London, United Kingdom.

Stewart Wolf, M.D., Director, Totts Gap Medical Research Laboratories, Bangor, Pennsylvania; Professor of Medicine, Temple University School of Medicine, Philadelphia, Pennsylvania.

GLOSSARY

In the discussions, a number of recently introduced or highly technical genetical terms were used. Definitions from a general biomedical viewpoint are provided.

BAYES' THEOREM

A theoretical procedure by which the ordinary probability is altered by knowledge of relevant outcomes leading to a conditional probability. For example, a woman who has a one-half chance of being a carrier for an X-linked disorder, *à priori*, has with each succeeding normal male child in the absence of any affected male child a decreasing conditional probability of actually being a carrier.

cDNA

cDNA is a *complementary DNA* copy of a specific messenger RNA (mRNA) or of that nucleotide sequence actually coding for a specific sequence of amino acids that forms a biological polypeptide. Originally, cDNA was produced by mRNA by the avian virus enzyme, reverse transcriptase. Such cDNAs can be cloned by recombinant DNA methods and can be used as probes for quantitating either mRNA content or chromosomal genes. The practical equivalent of a cDNA can now be obtained from appropriate clones of genomic DNA.

CHO

Chinese hamster ovary cells are a very well studied line that has been particularly suitable for somatic cell genetic studies.

CK - creatine kinase

The enzyme which produces the phosphagen, creatine phosphate, that in turn buffers the levels of ATP in cells, especially striated muscles. In vertebrates and humans, the general form of the enzyme is a *BB* dimer. In cardiac and developing skeletal muscles, the *MB* dimer is formed whereas in differentiated skeletal muscle, the *MM* dimer is found exclusively. Increased levels of CK activity are found in Duchenne muscular dystrophy patients, and in some of their family members as well as in other pathological and physiological states.

cM - centimorgan

Equivalent to one percent recombination of two genetic markers due to crossover between two homologous chromosomes during meiosis.

The term honors Thomas Hunt Morgan, the father of experimental genetics, who discovered genetic linkage and related it to chromosome location.

COLCHICINE

A drug that inhibits mitosis by disrupting the microtubules of the mitotic spindle. The compound binds specifically to the major protein subunit of microtubules, tubulin. Many other processes that require microtubules including the growth and function of nerves can be inhibited by colchicine. The drug is useful in eliminating symptoms of gout and Mediterranean fever.

CYTOCHALASIN B

One of a series of compounds that inhibit cytokinesis or the cleavage of cells in cell division. Cytochalasin B affects the polymerization of actin in many cells and *in vitro*.

FACS - fluorescence-activated cell sorter

A type of instrument using laser beams that detects the fluorescence or absence of fluorescence of individual microscopic particles and sorts them according to this detection. The particles may be previously separated by flow through a specific tube. Cells may be separated by their binding.

GENETIC MARKERS

A set of enzymes showing polymorphisms or clinical syndromes with inherited bases that are useful as markers of specific genetic loci on particular chromosomes.

ACP - acid phosphatase (autosomal, 11)
AD - adenosine deaminase (autosomal, 20)
ALD - adrenoleucodystrophy (X-linked)
APRT - adenine phosphoribosyltransferase (autosome)
CBD - colorblindness, deutan (X-linked)
CBP - colorblindness, protan (X-linked)
CGD - chronic granulomatous disease (X-linked)
GALA - α-galactosidase A (X-linked)
HEMA - hemophilia A (X-linked)
LDH - lactic acid dehydrogenase (autosomal, 11)
OA - optic atrophy (X-linked)
PFK - phosphofructokinase (autosomal, several chromosomes)
PGK - phosphoglycerokinase (X-linked)
PRPS - phosphoribosyl pyrophosphate synthetase (X-linked)
RS - retinoschisis (X-linked)
STS - steroid sulfatase (ichthyosis) (X-linked)
Xg(a)- blood group (X-linked)

G6PD - glucose 6-phosphate dehydrogenase

This is a key enzyme in the pentose phosphate shunt of glycolysis that produces NADPH. There are normal variants (A^+ and B) as well as clinically significant mutant enzymes. The locus of the enzyme is X-linked.

HeLa

A well studied human cell line originally derived from a carcinoma of a patient named Helen Lane. These cells are a common source of human DNA although their aneuploid nature suggests the possibility that the DNA may not be representative.

HPRT - hypoxanthine-guanine phosphoribosyltransferase

This enzyme is necessary for the salvage of these purine bases to produce the appropriate nucleoside monophosphates. There is a severe form of the enzyme deficiency in male children called Lesch-Nyhan syndrome, and a milder form in adults associated with gout. The enzyme locus is X-linked. Cells deficinet in HPRT are resistant to killing doses of 8-azaguanine.

kb - kilobases

One thousand base pairs or a multiple thereof of double-stranded DNA. May refer to bases in single-stranded DNA or RNA as well.

λ - bacteriophage lambda

A bacteriophage that has been widely used in genetics and molecular biology that becomes incorporated into the E. coli chromosome. Certain modified λ phages such as gtwes and Charon 4A and 21A are widely used in recombinant DNA work because they contain sites for specific endonucleases. Cosmids are modified λ phages that can accept pieces of DNA up to 40 kb.

LIBRARY

A collection of either genomic or cDNA sequences cloned in a particular vector that represents the genetic information of a species or the transcribed information of a cell population, respectively.

LOD

Logarithm (base 10) of the ratio of likelihood of one model to the likelihood of a second model (likelihood ratio). A LOD score of 0 is equal likelihood, of 3 is a 1000-fold ratio or "certainty" of one model over another. The LOD score is used in genetics as an index of linkage between two markers, that is the likelihood of finding the two linked over unlinked in a particular group of individuals.

LYONIZATION

An hypothesis suggested by M. Lyon to explain the observed pattern of X-linked gene expression of females and in males with multiple X chromosomes. Random inactivation of either X chromosome occurs in all cell lineages, leaving the other X active in terms of specific gene expression. Certain abnormal X chromosomes such as X-autosome translocations may remain active preferentially, and in those cases, the normal X is inactive.

μ, ν

The forward rates of mutation of gametes in males and females. Each character or gene may be assigned such a rate, and any set of genes may also have an average rate. Sites within genes may have different rates too. It is important to note that the rates of mutation in sperm or egg production may be different, and assumptions that they are the same may be significantly incorrect.

NICK TRANSLATION

A set of methods for radioactively labeling DNA molecules in vitro by the action of a DNA polymerase.

PLASMID

A circular double-stranded DNA molecule capable of autonomous replication in the cytoplasm of specific bacteria. Plasmids have been very useful in forming recombinant DNA molecules with DNAs from a variety of sources. pBR322 is a specific plasmid that can infect Escherichia coli which is widely used because of internal sites that can be cleaved by specific endonucleases, and drug resistant genes that permit ready selection.

REPETITIVE DEFICIENT

A series of cloned DNA sequences from human sources generated by Alu I action. Fragments so produced generally contain repetitive sequences about the Alu site. These fragments have been tested for absence of such repetitive sequences and may have been treated with

other restriction endonucleases to remove the repetitive sites. Blur 11 is a specific cloned Alu I-generated human DNA sequence.

RESTRICTION ENDONUCLEASES

Enzymes that make internal breaks at highly specific base sequences in double-stranded DNA. These sites of restriction are usually pallindromic in that the sequence on one strand is the reverse of the sequence on the second strand. Restriction enzymes are produced by specific strains of bacteria as protection against infection by viruses. Particular enzymes mentioned during discussions, their source and their specificity in terms of base sequence are as follows:

EcoRI	Haemophilis influenzae Rd	A↓AGCTT
Alu I	Arthrobacter luteus	AG↓CT
Bam HI	Bacillus amyloliquefaciens H	G↓GATCC
SAC I	Streptomyces achromogenes	GAGCT↓C
Msp I	Moraxella species	CC↓GG
Hpa I	Hemophilus parinfluenzae	GTT↓AAC
Taq I	Thermus aquaticus	T↓CGA
Bgl I	Bacillus globigii	GCC(N_4)↓NGGC (N = any base)
Bgl II	Bacillus globigii	A↓GATCT
Kpn I	Klebsiella pneumoniae	GGTAC↓C
*Mbo I	Moraxella bovis	↓GATC
*Sau 3A	Staphylococcus aureus 3A	↓GATC
Xba I	Xanthamonas badrii	T↓CTAGA

*Isoschizomers - Enzymes that can cut at the same sequence.

RFLP - restriction fragment length polymorphism

Inherited polymorphism or variant that alters the pattern of restriction endonuclease digestion of DNA. The mutation could affect the restriction site directly or cause an insertion or deletion in DNA near the site.

SOUTHERN

Refers to a procedure introduced by E. Southern of transferring pieces of DNA (usually generated by the action of specific endonucleases) from a gel electrophoretic separation to nitrocellulose paper. The resulting blot can be treated with radioactive DNA probes to detect the present of specific sequences within the generated fragments. In a Northern, RNA molecules are separated and then transferred. In a Western protein molecules are separated, transferred and identified by their reaction with specific antibodies.

The Benton-Davis and Grunstein-Hogness procedures use nitrocellulose paper to immobilize DNA from bacteriophage-induced plaques or plasmid-infected bacterial colonies, respectively, which are then treated with a specific readioactive DNA probe.

SV40 - Simian virus 40

A virus that can transform a variety of cell types and produce permanent cell lines that in animals form tumors. The chromosome of SV40 is very small, and the structure and function of its DNA has been determined in detail. Modified hybrids of SV40 and λ phage can be sued as universal vectors for DNA transfer, either into animal or bacterial host cells. The SV40 promoter or initiation site for mRNA synthesis can function with DNA inserts from other sources and lead to the synthesis of exogenous proteins in appropriate animal or bacterial hosts.

TK - thymidine kinase

The enzyme catalyzes the production of dTMP (deoxythymidine monophosphate) from thymidine and represents one pathway of dTTP (deoxythymidine triphosphate) biosynthesis, necessary for DNA synthesis and cell division. Cells deficient in this enzyme are resistant to killing doses of BrdU (5 bromodeoxyuridine), a thymidine analogue. TK^- cells are frequently used in genetic and transfection experiments with BrdU as the selecting drug.

VECTOR

An agent consisting of a DNA molecule that can replicate automomously the host cell's chromosomes and to which another DNA segment may be attached and also be replicated.

WALKING

The spanning of large distances of chromosomal DNA by the molecular overlapping of cloned DNA sequences.

Xp21

X chromosomes, short arm, band 2, subband 1. The same formalism could be used for other chromosomes and their regions. For autosomes a number instead of X would be used. p or q refer to short or long arm. The band and subband designations are usually the result of high resolution prophase chromosome band patterns following trypsin treatment and Giemsa staining.

CONTENTS

CHAPTER 1

PREVALENCE AND HERITABILITY OF DUCHENNE MUSCULAR DYSTROPHY

DR. P. MICHAEL CONNEALLY: Duchenne muscular dystrophy is inherited as an X-linked recessive disorder. The lesion seems to be in the middle of the short arm of the X chromosome. There is also an autosomal recessive condition which is very akin to Duchenne and is relatively common among the Amish (1). For practical purposes, Duchenne dystrophy occurs only in males. Although cases of females with Duchenne muscular dystrophy have been reported (2), the majority are Turner's syndrome (XO) or structural abnormalities of the X. In fact, you will see stated in textbooks that the frequency of Duchenne muscular dystrophy in Turner's is the same as the frequency in males because they only have one X chromosome. This is not quite true. If, for example, all of the nondisjunction occurs in the male, then the stated frequency in Turner's syndrome would be correct. If, on the other hand, all of the nondisjunction occurred in the female, then all of the X's would come from the male; in this case you would have no Duchenne since it is an X-linked lethal. There is also a possibility of extreme lyonization which could also cause Duchenne in females if, by chance, all or the vast majority of their normal X's are inactivated (2).

Figure 1 shows a map of the X. The Duchenne gene is generally thought to be on the short arm of the X and it is not closely linked to any of the known X-linked markers; for example, the Xg blood group. Duchenne is one of the most common X-linked disorders with a frequency of at least 1 in 4800 males.

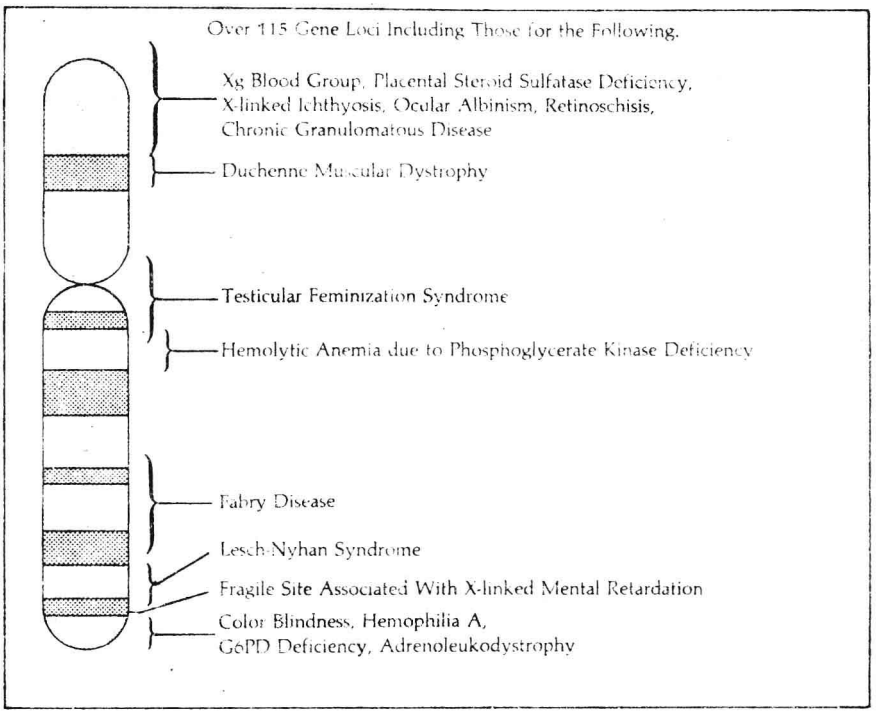

Figure 1: Linkage map of the X chromosome. From: McKusick, V.A., The Anatomy of the Human Genome; Hospital Practice, p. 85, 1981.

Carrier Females

There are a number of ways to determine the frequency of carrier females in the population. One way to become a carrier for the Duchenne gene is through a mutation in the parental sperm at a rate per gamete in males (μ) or in the egg at rate per gamete in females (ν). The overall mutation rate to produce a carrier female is $\mu + \nu$. The mutation could have occurred in her mother's father or in her mother's mother and then be transmitted to her at a rate of $1/2\ (\mu + \nu)$. The mutation could also have occurred one generation previous to this with a probability of one-fourth that it is transmitted two generations, etc. The overall probability that the woman is a carrier is $(\mu + \nu) + 1/2\ (\mu + \nu) + 1/4\ (\mu + \nu) + 1/8\ (\mu + \nu)$, etc. This is the sum of an infinite number of terms of a geometric series and becomes $2\ (\mu + \nu)$ (3).

If the mutation rate is the same in males and females, $(\mu = \nu)$, then the frequency of carrier females in the population

is 4μ. In carrier females one-half of the Duchenne genes are new mutations and a further one-fourth are only one generation "old." For example, $(1/2)^{10}$ or only one-tenth of one percent have existed ten or more generations.

There are two ways to get an affected male: one from a carrier female and the other from a new mutation. The chance that the mother is a carrier is 4μ and the chance of transmitting the gene to her son is 2μ. Adding the probability of a new mutation, μ, the frequency of carrier males in the population becomes 3μ. This is called Haldane's rule, after J.B.S. Haldane, the great English population geneticist (4). Assuming a stable frequency of affected males and recognizing that they will not reproduce, one-third of all cases of Duchenne would be new mutations, and two-thirds would be due to preexisting genes. Therefore, a substantial number of mothers of affected individuals should themselves not be carriers, and their recurrence risk is essentially zero, i.e. the risk should only be the mutation rate. The frequency of cases of DMD is 1 in 4800, and theoretically this is three times the mutation rate. Therefore, dividing by 3, we find a mutation rate of 1 in 15,000 or approximately 7 in 100,000. That is a very high mutation rate, higher than most other estimated mutation rates in man.

Genetic Heterogeneity and Mental Retardation

What is the most plausible explanation for the high frequency of the disease if one does not accept this high mutation rate? One possible reason is genetic heterogeneity, that is more than one locus is involved in Duchenne. The prime example of such a hypothesis is xeroderma pigmentosum where there are 7 or 8 complementation groups with distinct genetic lesions known in most of them. Clinical heterogeneity may be found in Duchenne. If a patient is severely mentally retarded and has an affected sib, his sib will also be retarded. Normal and retarded Duchennes are not found in the same family (5). This suggests that there is genetic heterogeneity, expressed as Duchenne with and Duchenne without severe mental retardation.

DR. BROOKE: We have been studying 150 boys with Duchenne dystrophy followed in four separate university clinics. Apart from the Becker type which we separated out, we found interesting evidence of heterogeneity unrelated to IQ. We called these cases outliers because they are doing better than the others and not deteriorating as rapidly. We identify them by the strength of the neck flexors and by the creatine in the urine, but not by the IQ. We found mixtures in the same family of bright and dull, instances where, for example, one Duchenne child is living with the father and the other is living with the mother and in that situation we see differences in IQs. We see no correlation between IQ and muscle strength in our series.