TIME BOMBS IN THE HUMAN GENOME: EXPLODING TRIPLETS THAT CAUSE DISEASE

Medical Grand Rounds
Parkland Memorial Hospital
University of Texas Southwestern Medical Center
March 11, 1993

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Even before the first geneticist sketched the first family tree, astute physicians noted that certain familial diseases grew progressively worse in each succeeding generation (reviewed in [1]). These diseases also caused symptoms earlier in affected offspring than in the affected parent. The physicians coined a term for this phenomenon: "anticipation". In the middle of the 20th century "genetic anticipation" fell into disrepute. There was no precedent in plants or lower animals, and geneticists concluded that anticipation was a figment of the physicians' imagination.

The past two years have witnessed a dramatic turn-about in this view. The physicians were right. Genetic anticipation happens and we know how. And in learning the mechanism we have uncovered a new type of plasticity in the human genome with implications for many hereditary diseases.

These remarkable discoveries emerged from studies of three genetic diseases that show anticipation. They are: Myotonic Dystrophy, the Fragile X Syndrome, and X-linked Spinal and Bulbar Muscular Atrophy (Fig. 1). Today I will review what we've learned about anticipation

THREE DISEASES WITH GENETIC ANTICIPATION

Myotonic Dystrophy
Fragile X Syndrome
X-linked Spinal and Bulbar Muscular Atrophy

FIG. 1

from each of these diseases and then I'll present some general conclusions and speculations.

Let's begin with **Myotonic Dystrophy** the first disease in which genetic anticipation was documented clinically (1a).

Fig. 2 shows the title of a 1948 paper by L.B. Penrose, the famous British geneticist,

REPRINTED FROM *ANNALS OF EUGENICS*, VOL. 14, PART 2, pp. 125–132, 1948

PRINTED IN GREAT BRITAIN

THE PROBLEM OF ANTICIPATION IN PEDIGREES OF DYSTROPHIA MYOTONICA

BY L. S. PENROSE

FIG. 2

which buried the notion of genetic anticipation for forty years.(1) Fig.3 shows his conclusion. Why was Penrose wrong? Let's look at his paper.

The tendency for anticipation to occur in pedigrees of hereditary disease is due to the manner of their selection and is not a phenomenon of direct biological significance. The careful analyses

FIG. 3

Penrose assembled a list of seven medical conditions that show genetic anticipation (Fig. 4) and compared the mean age of onset in parent-child pairs. The most striking finding

Table 1. *Statistics of age of onset in years**

Type of disease	Number of parent and child pairs (N)	Mean values			General standard deviation σ	Index of anticipation $D/2\sigma$	Parent-child correlation
		Parent	Child	Difference (D)			
Peroneal atrophy (dominant)	86	24·30	19·36	4·94	13·59	0·18	0·76
Muscular dystrophy (dominant)	90	27·44	21·00	6·44	13·08	0·25	0·62
Hereditary glaucoma	113	42·08	30·66	11·42	17·99	0·32	0·81
Huntington's chorea	153	40·80	31·98	8·82	12·38	0·36	0·59
Diabetes mellitus	216	60·29	43·06	17·22	15·26	0·56	0·44
Mental illness (all diagnoses)	1728	50·50	34·20	16·30	16·80	0·48	0·38
Dystrophia myotonica	51	38·48	15·24	23·24	12·92	0·90	0·32

* The figures for mental illness and for diabetes mellitus are derived from data collected by the present writer. All others are derived from *The Treasury of Human Inheritance* (Bell, 1932, 1934, 1935, 1943 and 1947).

FIG. 4

was in dystrophia myotonica (myotonic dystrophy), a dominant trait. The mean age of onset was 38 years in the parent and only 15 years in the child, a difference of 23 years. Several other diseases also showed significant genetic anticipation. I call your attention to Huntington's chorea and diabetes mellitus. I'll return to these at the end of my talk. Using myotonic dystrophy as an example, Penrose performed a sophisticated mathematical calculation proving

conclusively that genetic anticipation was an artifact. Fig. 5 shows this sophisticated

Sib

Sib: Onset age	15	45	Total
45	$\frac{pq}{4}$	$\frac{pq}{2}+q^2$	$q-\frac{pq}{4}$
15	$p^2+\frac{pq}{2}$	$\frac{pq}{4}$	$p-\frac{pq}{4}$
	$p-\frac{pq}{4}$	$q-\frac{pq}{4}$	$1-\frac{pq}{2}$

$$r=\frac{8+3pq}{(4-p)\,(4-q)},$$

which rises to a maximal value of 0·71, when $p=q=\frac{1}{2}$.

FIG. 5

mathematical calculation. The flaw must be obvious to all of you, so I won't point it out. Penrose was wrong. Myotonic dystrophy does show genetic anticipation, and the reason is conceptually simple although mechanistically difficult. Before getting to the genetics, I will briefly review the clinical problem of myotonic dystrophy.

Fig. 6 shows the facial features of an adult patient with this progressive muscular disease (2). The disease affects the facial muscles and this produces temporal wasting and a sunken appearance together with ptosis and an apathetic expression.

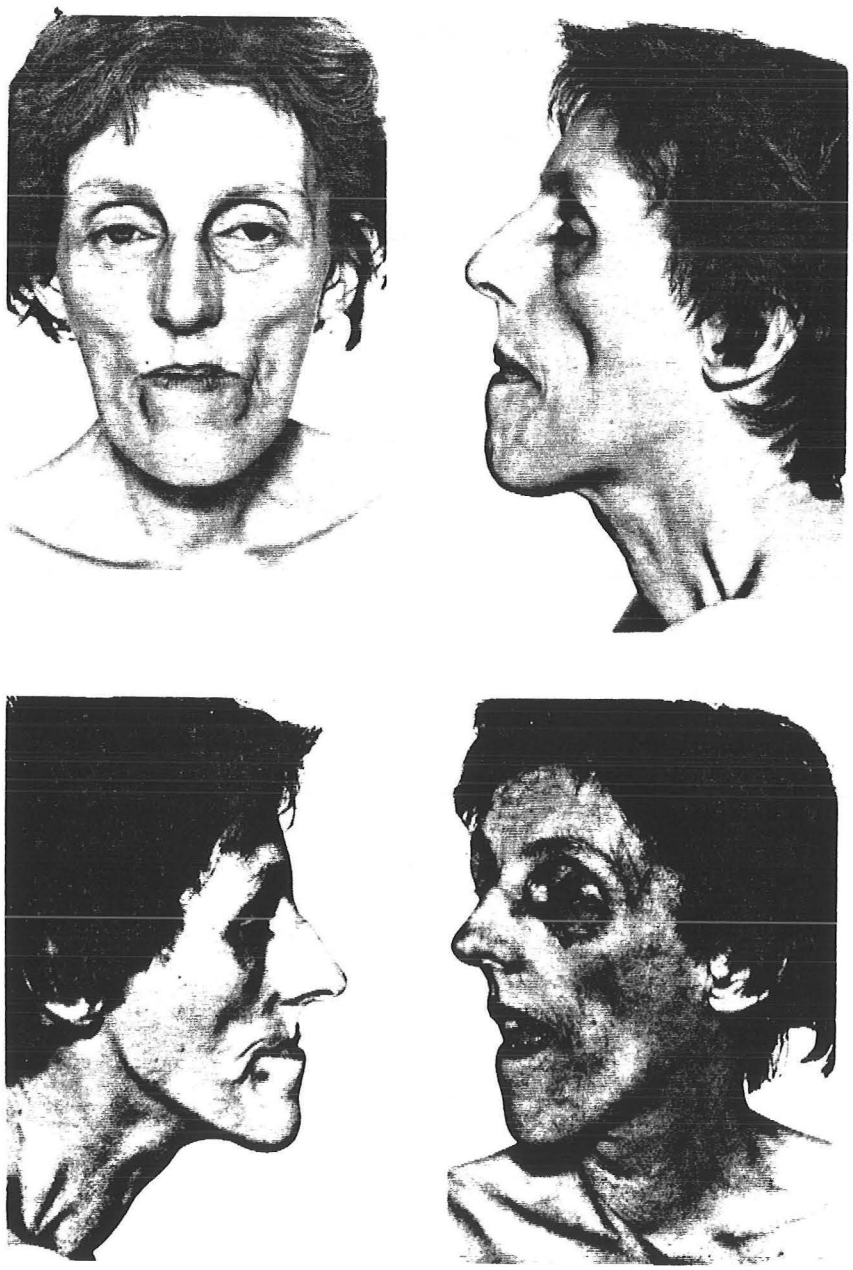


FIG. 6

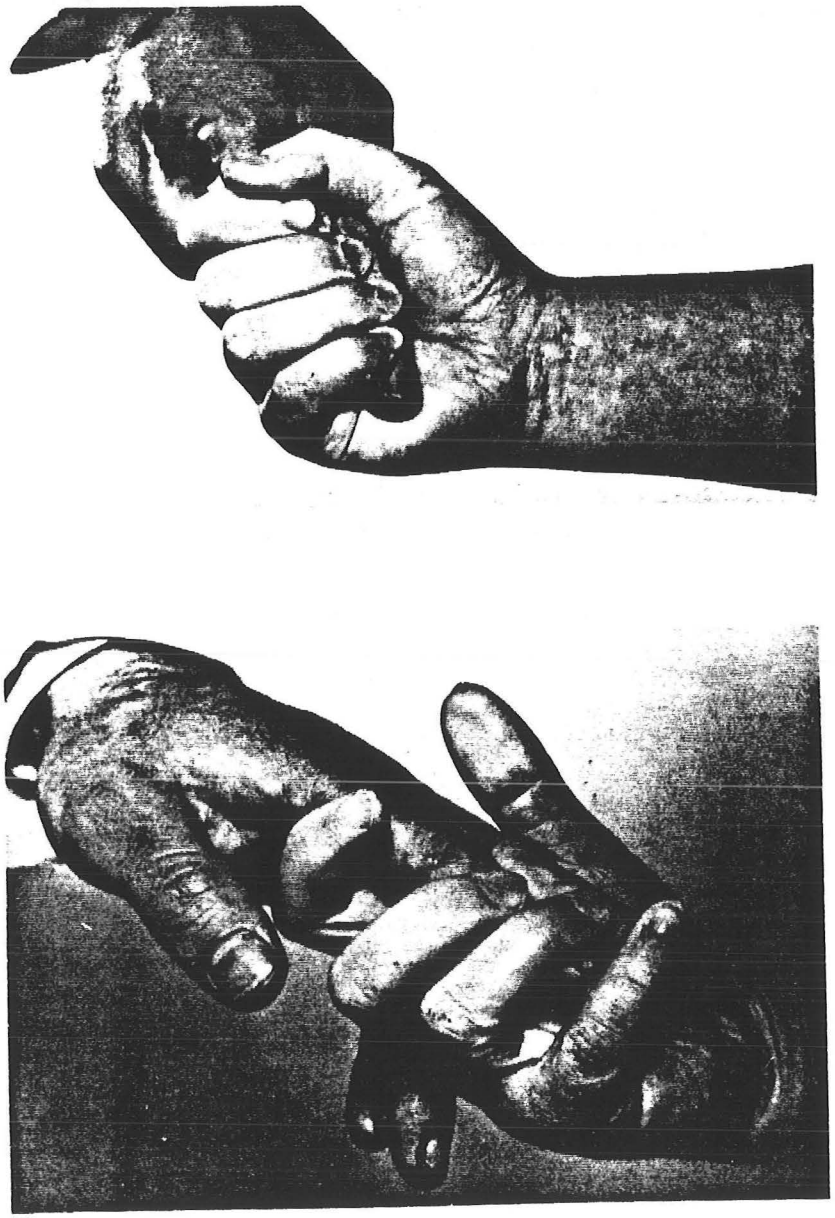
Fig. 7 lists the muscles most prominently affected (2). Myotonic dystrophy is unusual in affecting the distal muscles of the forearm and leg most prominently. The palate and pharyngeal muscles are also affected, leading to difficulty in swallowing and death by aspiration.

Table 2.1 Muscular involvement in myotonic dystrophy.

Muscles most prominently affected
Superficial facial muscles
Levator palpebrae superioris
Temporalis
Sternomastoids
Distal muscles of forearm
Dorsiflexors of foot
Other muscles commonly affected
Quadriceps
Diaphragm and intercostals
Intrinsic muscles of hands and feet
Palate and pharyngeal muscles
Tongue
External ocular muscles
Muscles frequently spared
Pelvic girdle
Hamstrings
Soleus and gastrocnemius

FIG. 7

Fig. 8 shows the hallmark of the disease, myotonia (2). When the patient grips an object firmly and is then asked to relax the grip, relaxation is markedly delayed. Electromyography shows that this is due to electrical activity that persists even after the neurologic stimulus has ceased. Myotonic dystrophy is not the only cause of myotonia, but it is the only adult disorder in which myotonia leads inexorably to death, and in which multiple systems are involved (see below).



FIG, 8

Fig. 9 shows the muscle histology in this disease (2). As shown in panel A there is a loss of uniformity of muscle fibers, and widespread atrophy. Panel B shows chains of nuclei in the center of muscle fibers which is distinctly abnormal although not pathognomonic.

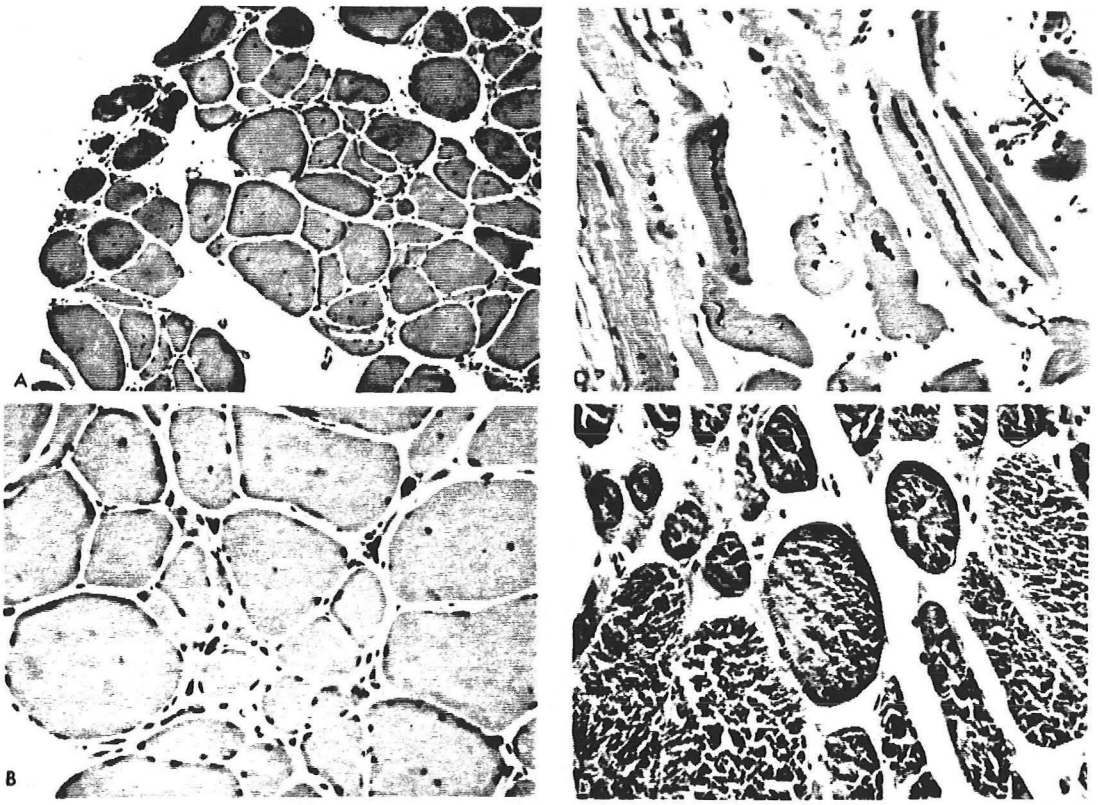


FIG. 9 | Changes in muscle histology in adult myotonic dystrophy. *A.* Transverse section showing variation in fiber size and numerous internal nuclei. *B.* Higher power transverse section showing atrophic fibers with clumped nuclei. *C.* Longitudinal section showing long chains of internal nuclei. *D.* Transverse section showing ringed fibers. (*From Harper.*[180])

Myotonic dystrophy is a multi-system disease. Fig. 10 lists the organ systems that are often involved (2). I draw your attention to smooth muscle which is affected as well as skeletal

Table 2.3 Systemic involvement in myotonic dystrophy.

System	Principal involvement
Smooth muscle	Oesophagus, colon, uterus (other sites may also be affected)
Heart	Conduction defects, in particular heart block, atrial arrhythmias; less commonly, cardiomyopathy
Lungs	Aspiration pneumonia from oesophageal and diaphragmatic involvement, hypoventilation
Peripheral nerve	Variable and rarely clinically significant; minor sensory loss may occur
Brain	Severe involvement in congenital form; mild mental deterioration frequent in adults; hypersomnia
Endocrine	Testicular tubular atrophy; diabetes (rarely clinically significant); sometimes abnormalities of growth hormone and other pituitary functions
Eye	Cataract, retinal degeneration, ocular hypotonia, ptosis, extraocular weakness
Skeletal	Cranial hyperostosis, air sinus enlargement; jaw and palate involvement; talipes (childhood cases); scoliosis (uncommon)
Skin	Premature balding; calcifying epithelioma

FIG. 10

muscle. The heart shows conduction defects leading to heart block and arrhythmias. The brain is severely involved in the congenital form of this disease. However, even in adults mental deterioration is frequent. The endocrine abnormalities have long been of interest. There is a high incidence of insulin resistance leading in some cases to glucose intolerance and even to frank diabetes. The eye is affected by cataract in the majority of the adults cases. Retinal degeneration is less well known and less common, but is well documented. It involves both the peripheral and central retina. In the skin premature balding occurs.

Fig. 11 shows the radiating cataracts that are seen early in this disease (3). Usually they are picked up by slit lamp examination. Eventually they lead to complete opacification of the lens. In some patients, cataract may be the sole clinical manifestation without detectable myotonia or muscle weakness.

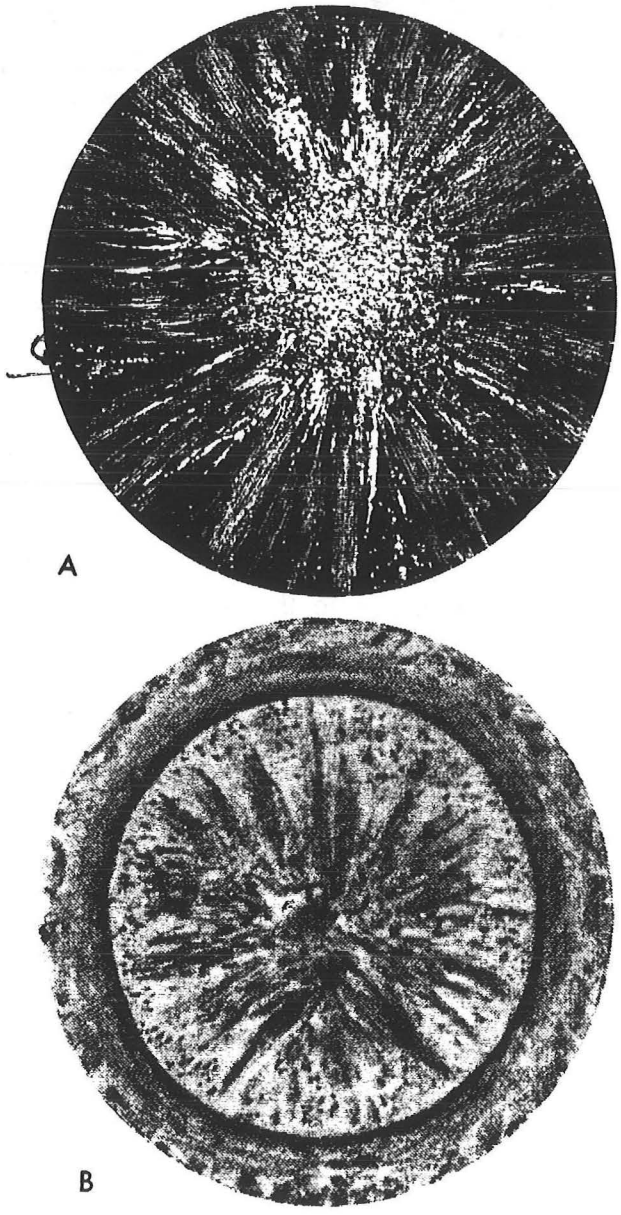


FIG. 11

Fig. 12 shows the appearance of an infant with the severe congenital form of myotonic dystrophy (3). These infants have profound mental retardation and muscle weakness. The disease is much more severe and rapidly progressive than is the adult onset form. Yet numerous genetic studies show that the congenital form occurs in families where previous generations had the adult form. This was one of the first clues to genetic anticipation in this disease.

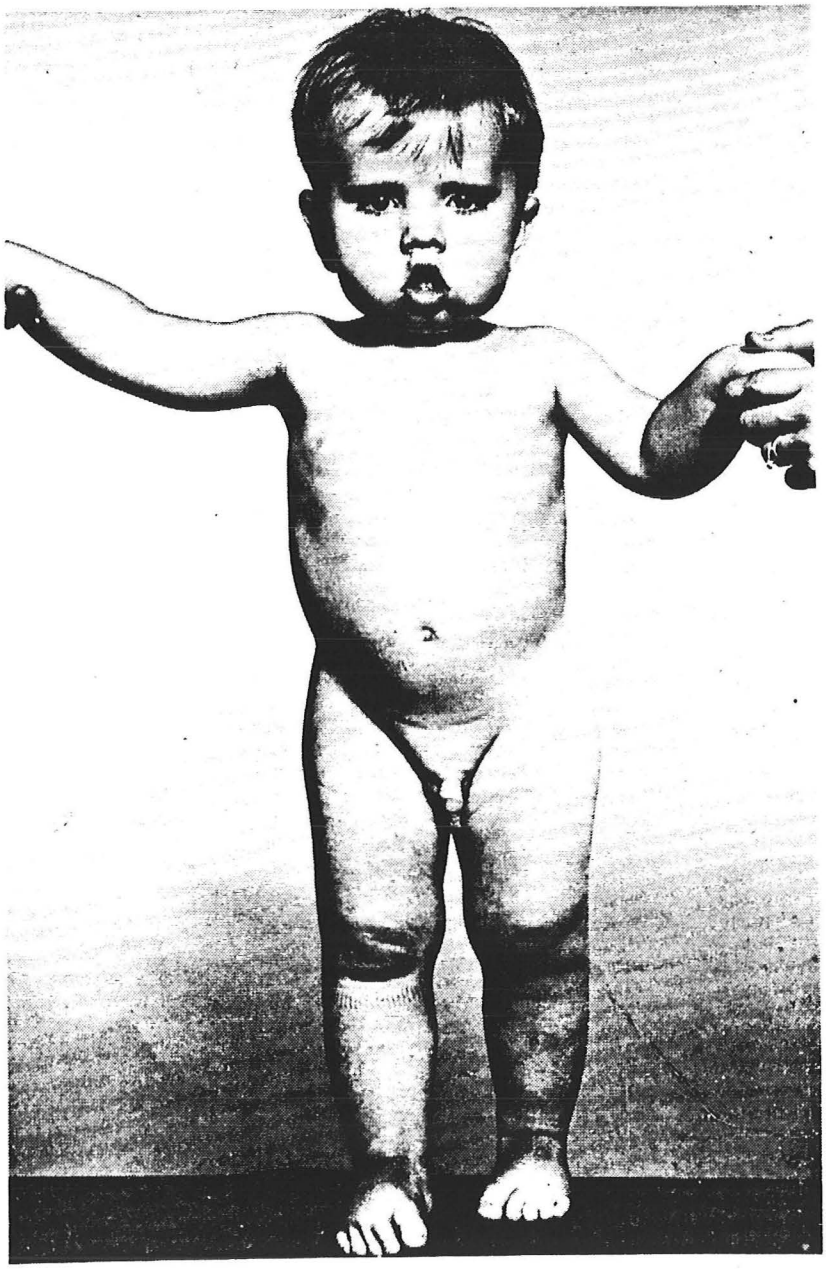


FIG. 12

Fig. 13 shows that more than half of the patients studied have an elevated insulin response to glucose, indicating insulin resistance (3). However, less than 20% of the patients had a diabetic glucose pattern, and only two had clinical diabetes. We conclude that insulin resistance is a definite part of the syndrome, and that most patients are able to compensate for this by enhanced insulin secretion without developing diabetes.

Table 6.8 Abnormal glucose tolerance in myotonic dystrophy.

	Total	Clinical diabetes	Diabetic GTT	Insulin hyperresponsive
Walsh *et al.* (1970)	20	0	2	19
Huff *et al.* (1967)	6	0	0	6
Barbosa *et al.* (1974)	29	2	9	21 (out of 26)
Poffenbarger *et al.* (1976)	8	0	3	5
Bird and Tzagournis (1970)	10	0	2	4
Bjorntorp *et al.* (1973)	17	0		
Cudworth and Walker (1975)	10	0	1	6
Gorden *et al.* (1969)	12	0	0	7
Mendelsohn *et al.* (1969)	11	0	3	1
Total	123	2	20	69 (110 studied)

FIG. 13

Fig. 14 shows the age of onset (3). About 10% of the affected individuals have the disease at birth. Thereafter, the percentage of patients showing clinical symptoms increases steadily over a fifty year period. This pattern is extremely unusual for a genetic disease and it suggests an unusual mode of inheritance.

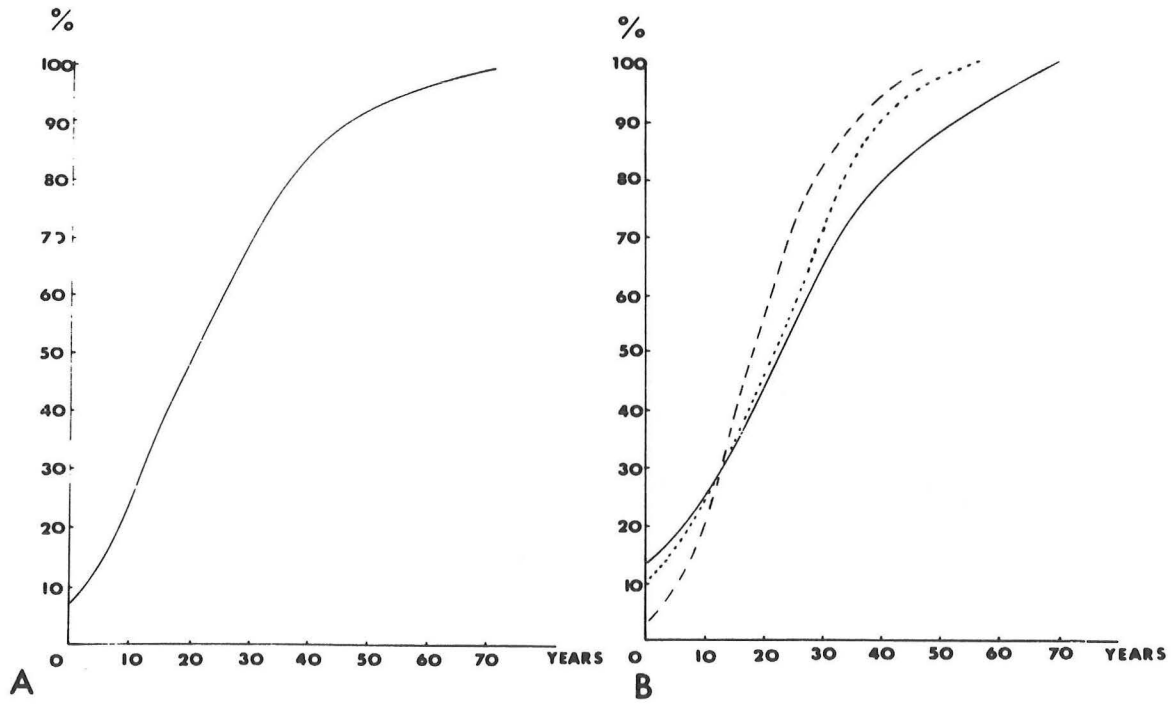


FIG. 14

Fig. 15 shows a typical pedigree for myotonic dystrophy (2). The solid symbols indicate affected individuals. The bold number to the upper right indicates the decade in which the disease first appeared. The great-grandfather developed the disease in the seventh decade. His two daughters became ill in the fourth decade. One daughter had four affected children each of whom developed the disease in the first or second decade of life. One of the daughters had an infant son who had the severe congenital form of the disease. This pedigree illustrates dramatically the earlier onset in each generation that is the hallmark of genetic anticipation. Pedigrees like this are the rule rather than the exception in myotonic dystrophy.

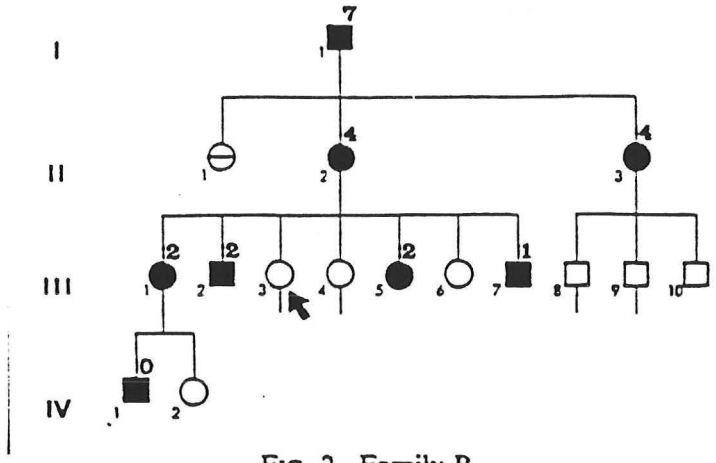


FIG. 15

FIG. 2. Family B.

Mendel never saw anything like this! How can genetic anticipation be explained? Mutations don't get progressively worse. Or do they?

The first step in solving this problem came with the localization of the mutant gene to human chromosome 19.

Fig. 16 shows a map of our favorite chromosome (2). Sitting majestically on the short

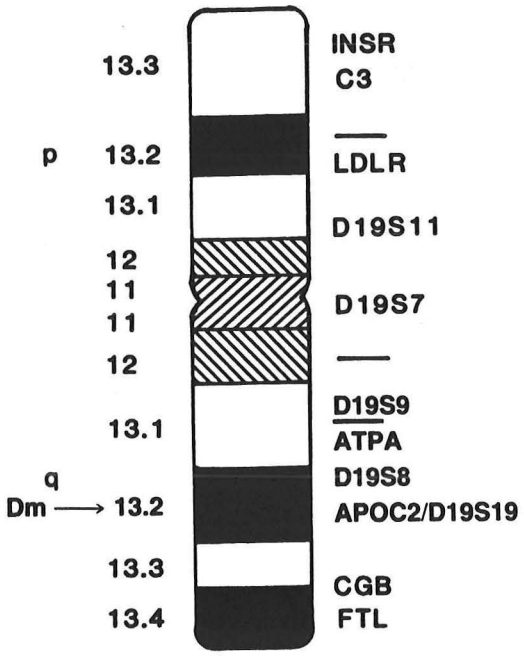


FIG. 16

arm is the LDL receptor. The myotonic dystrophy locus is on the long arm near the gene for apo C2, another player in lipoprotein transport. This linkage is pure chance and does not imply a functional relationship.

The breakthrough in discovering the genetic defect in myotonic dystrophy came last year when several groups isolated this region of chromosome 19 (4-10). They cloned human chromosomes by inserting them as artificial chromosomes in yeast. These are called yeast

artificial chromosomes (YACs for short). Fig. 17 shows the map locations of a series of YAC fragments that span the region of the myotonic dystrophy locus (4). Using these fragments as probes several groups demonstrated an abnormality on chromosome 19 in patients with myotonic dystrophy (4-10). Patients with the most severe form of the disease had an enlargement of one region suggesting that there was extra genetic material at this point. In order to trace this down, the research groups turned to another powerful technique that allows one to examine minute areas of the genome. That technique is known as the polymerase chain reaction.

I hope that everyone understands the concept of PCR because it is among the most powerful techniques in genetics. Briefly, PCR allows scientists to produce an infinite number of copies of any small region of the human genome by biochemical means without any cloning.

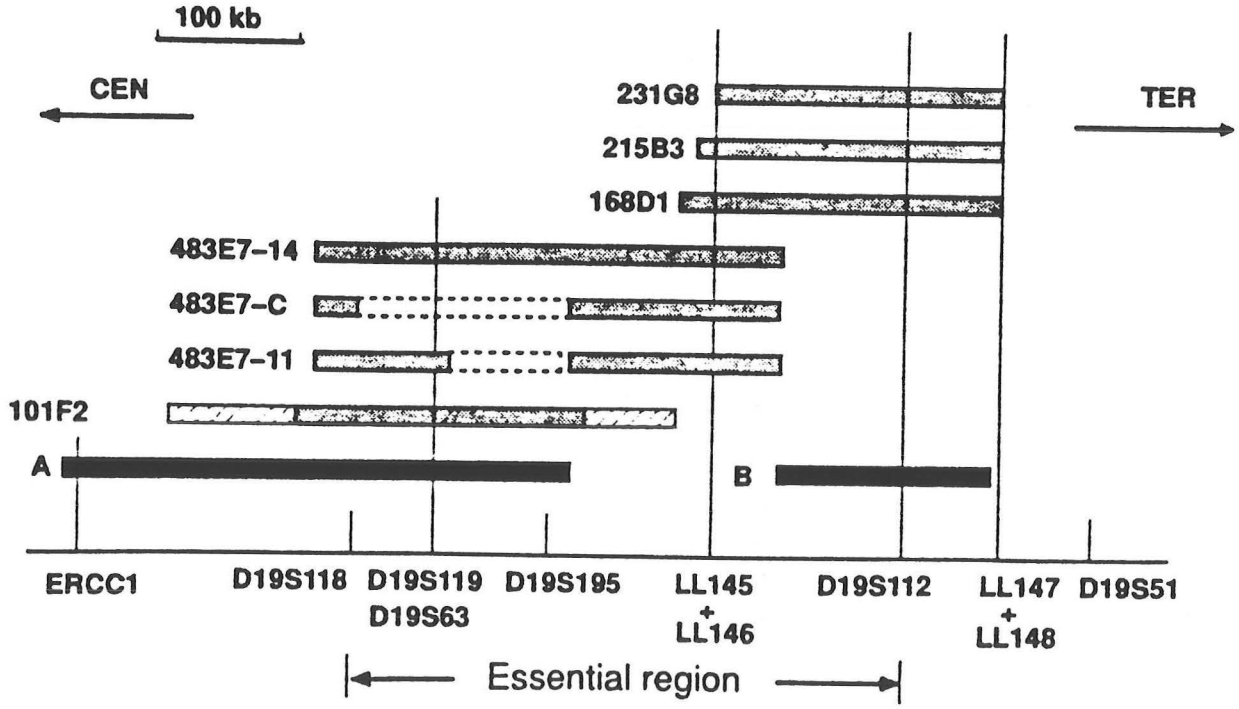


FIG. 17

Fig. 18 explains the PCR technique (11). Briefly, one can take any piece of double-stranded DNA and denature it, which means to separate the strands by heating. The piece of DNA need not be pure. In fact, it can be the total DNA in a cell-free homogenate. The separated strands are allowed to hybridize or anneal to short oligonucleotide primers which form base pairs with the opposite ends of each strand. One then adds DNA polymerase and the four deoxynucleotide triphosphates which extends the primers using each strand as a template. We have now duplicated both strands of the original DNA. If we repeat the cycle we can convert these two copies into four, eight, sixteen and so forth. With repeated cycles we can produce millions of copies of this sequence and then we can study the DNA segment that we have reproduced.

The beauty of PCR is that it can be applied to DNA extracted from whole cells. No purification is required. One can therefore study any region of any gene from any individual provided only that you know sufficient sequence to make complimentary oligonucleotide primers.

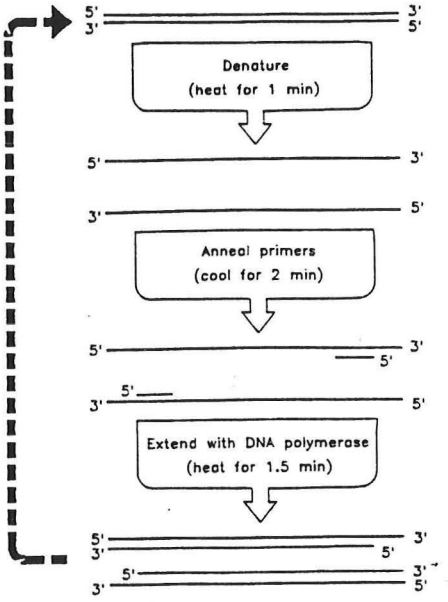


FIG. 18

Last year workers in three laboratories used PCR to amplify the abnormal region of chromosome 19 from myotonic dystrophy patients (7-10). Fig. 19 shows the sequence of this region taken from the paper by Houseman and colleagues, who published the most complete paper of the three that were rushed to publication (8). The sequence of this region from a normal individual, is shown in Fig. 19. The sequence contains a sequence of three nucleotides (CAG) that is repeated five times. On the other strand, reading from the 5´ to 3´ direction, the sequence is CTG. Most people now refer to this sequence as the CTG repeat.

FIG. 19

To learn the reason for the increased size of this fragment in MD patients Houseman and colleagues performed PCR with the two oligonucleotide primers designated 101 and 102 that were designed to copy the segment that contains the triplet repeat.

Fig. 20 shows the results of this PCR performed with DNA from normal individuals (8). Each lane shows the results with genomic DNA from a single individual. You will notice that fragments of many different sizes were found. For each individual, either one or two bands is visible. The scientists determined the nucleotide sequence of these PCR products and found that the difference in size was explained by a difference in the number of triplet CTG repeats, as shown on the vertical axis. Recall that the normal sequence contained five repeats. Indeed, most normal individuals have at least one gene with five repeats. However, many of the genes in the population have a larger number of repeats, extending up to 24. Many individuals have two different bands indicating that they have inherited two different genes with unequal numbers of repeats, one from each parent. This gene is therefore polymorphic. Most individuals are heterozygous for two different numbers of repeats. But notice, normal individuals never have more than 30 repeats.

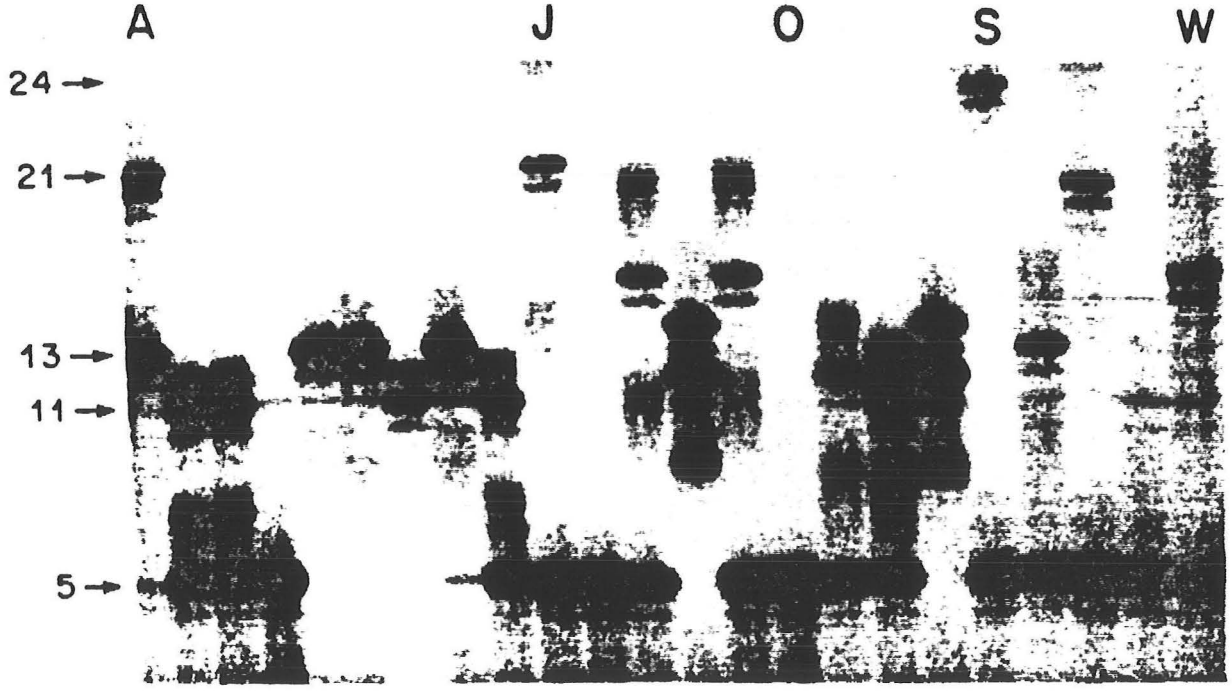


FIG. 20

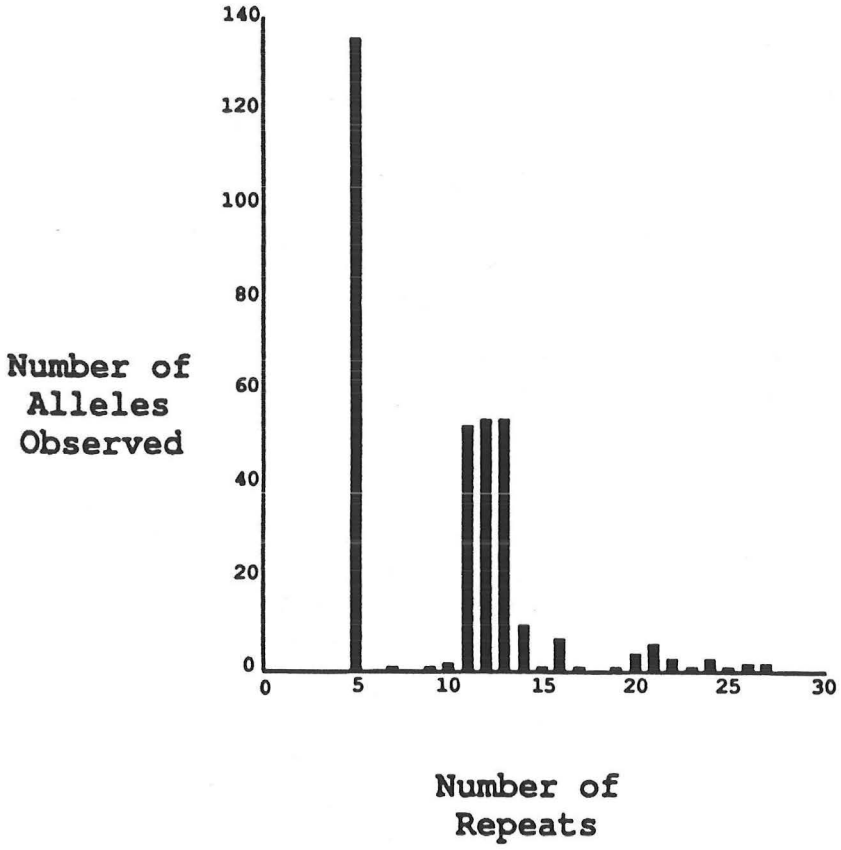
Fig. 21 shows the frequency distribution of the repeats among normal subjects (8). Five is the most usual number. However, many chromosomes have ten or more copies of the CTG repeat. Again note that no normal chromosome has more than 30 copies.

Contrast this with the situation in myotonic dystrophy. Fig. 22 shows PCR products from two normal individuals (lanes 2 and 3) and two grandparents of myotonic dystrophy patients (lanes 1 and 4) (8). You can see both MD ancestors have a band that is much larger than normal and contains 52 repeats. Both of these individuals had extremely mild manifestations of the disease, but their grandchildren had severe manifestations. The lesson is clear. If you have 52 copies of the CTG repeat your grandchildren will have severe myotonic dystrophy. How can this be explained?

Fig. 23 shows four generations of a family with myotonic dystrophy (8). The great-grandmother (Subject I-1) had no symptoms other than cataracts. PCR showed that she had one abnormal gene whose amplified product was about 300 base-pairs long. The amplified product includes about 80 base-pairs of DNA that flanks the repeats). This woman therefore hads about 70 triplet repeats. The other copy of the gene had a PCR product of 120 base-pairs. Subtracting the 80 base-pair flanking sequence this indicates about 12 repeats, which is normal. In the second generation the subjects with open symbols had no symptoms. Individual 2-4, like his mother, had cataracts as the only sign of potential myotonic dystrophy. In the son of individual II-4 something strange happened. This individual inherited a normal chromosome with a 120 base-pair amplified band from his mother. From his father he inherited a chromosome in which the size of the amplified fragment had increased dramatically to 2,000 base-pairs. The number of triplet repeats had increased from 70 to 700. He had classical adult onset myotonic dystrophy with multiple system involvement. Subject III-1 had a son, IV-2, who inherited the gene with the 2 kilobase fragment. He developed myotonic dystrophy even earlier than his father, namely, in the teenage years. In this family genetic anticipation was associated with a dramatic increase in the number of triplet repeats. The great-grandmother (1-1), and her son (2-4) can be considered to have a "premutation". They have a slightly increased number of triplet repeats (i.e.,40), but they have only very mild disease. However, the increased number of repeats establishes a dangerous situation. Once the threshold of 30 repeats has been exceeded subsequent generations may inherit a huge number of repeats, and myotonic dystrophy will develop early in life. The progressive amplification of triplet repeats neatly explains genetic anticipation.

Within the last month we have learned that repeats can not only expand: they can also contract. Two papers in the New England Journal (12,13) showed mutations that reversed themselves. The most impressive paper was from a group in Nijmegen, Holland who devised a technique that permits precise sizing of very large repeats after PCR amplification (12).

FIG. 21


Number of Alleles Observed
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Number of Repeats

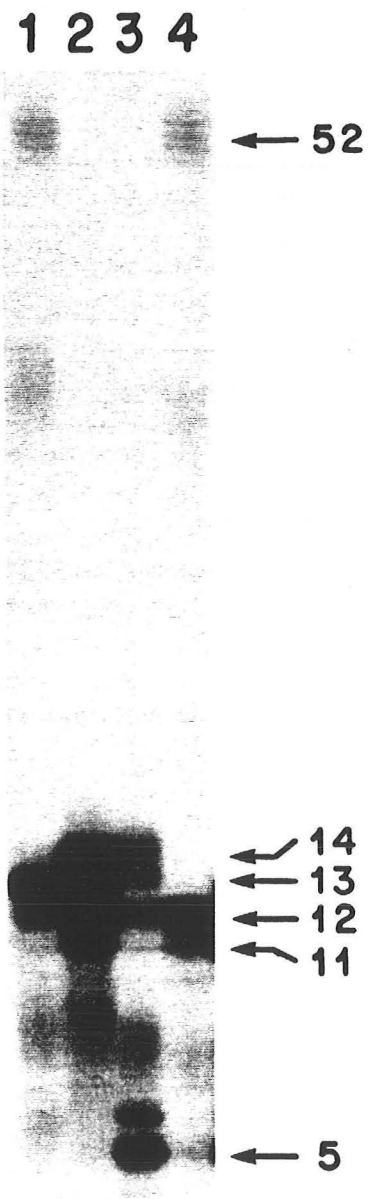

1 2 3 4
52
14
13
12
11
5

FIG. 22

Fig. 24 shows two families from the Dutch paper. In Family 1, two brothers (subjects 2-2 and 2-3) both developed myotonic dystrophy in their twenties. PCR showed a marked increase in the repeats, which ranged between 200 and 600. Prior to the discovery of the CTG repeats, the Dutch geneticists had used ordinary linkage analysis to identify the mutant chromosome in this pedigree. They used two genetic markers designated apo C2, and X75B, which flank the muscular dystrophy gene, and are very closely linked to it. In this family the mutation is on a chromosome that contains allele 3 at the apo C2 site and allele 1 at the X75B site. This chromosome is shown in a box for Family 1. The wife of subject 2-3 became pregnant three times and she sought prenatal diagnosis. The first fetus had chromosome 3-1, which was considered to be the muscular dystrophy chromosome. The pregnancy was terminated by abortion. Later, after the existence of the CTG repeat was recognized DNA from the fetus was subjected to PCR amplification which confirmed that the fetus had inherited the abnormal gene. A second pregnancy terminated in a spontaneous miscarriage. The fetus did not have the abnormal chromosome, and there was no tri-nucleotide expansion. The shocking finding came in the third pregnancy. This fetus was subjected to pre-natal diagnosis which showed that she had indeed inherited the 3-1 chromosome, which was supposed to contain the mutation. Ordinarily, the pregnancy would have been terminated by abortion. By now, however, the existence of the CTG repeat had been recognized. Therefore, DNA from the chorionic villus biopsy was subjected to PCR analysis. Shockingly, the DNA did not contain the abnormally enlarged triplet repeat. The fetus was born as a healthy infant. Afterwards, DNA was obtained from several tissues. The authors could find no evidence of an abnormal amplification of the CTG repeat in DNA from any of her tissues. They confirmed that she had inherited the abnormal chromosome, but the number of repeats had shrunk to 24. A similar situation is shown in Family 2. Subject 2-2 inherited the abnormal chromosome from his father but the amplification disappeared.

These findings introduce a new word of caution into pre-natal diagnosis and genetic prediction. In diseases caused by triplet expansion, linkage analysis is not sufficient to predict whether an individual is affected. Direct measurement of the CTG repeat is necessary.

These findings show that the mechanism that expands repeats must be reversible. Although several cases of this contraction have been reported, the phenomenon cannot be all that frequent. The classic studies of myotonic dystrophy show a very high penetrance, that is, about 50% of all at-risk relatives will show some sign of the disease (3). If triplet contraction were frequent one would expect to see a lower than expected number of affected relatives.

The data from the Dutch group (12) illustrates another important point about triplet expansion. Affected individuals have an enormous range of fragment sizes. The number of triplets extends from 200-600. This DNA was obtained from blood lymphocytes. Once the number of triplets has expanded past a threshold at meiosis, the number can continue to expand at mitosis. This gene is as unstable in somatic cells as in germ cells.

The contraction of the repeats raises another point. As I told you, in Family 1 the number of repeats has decreased to 24, which is clearly a normal number. But is this

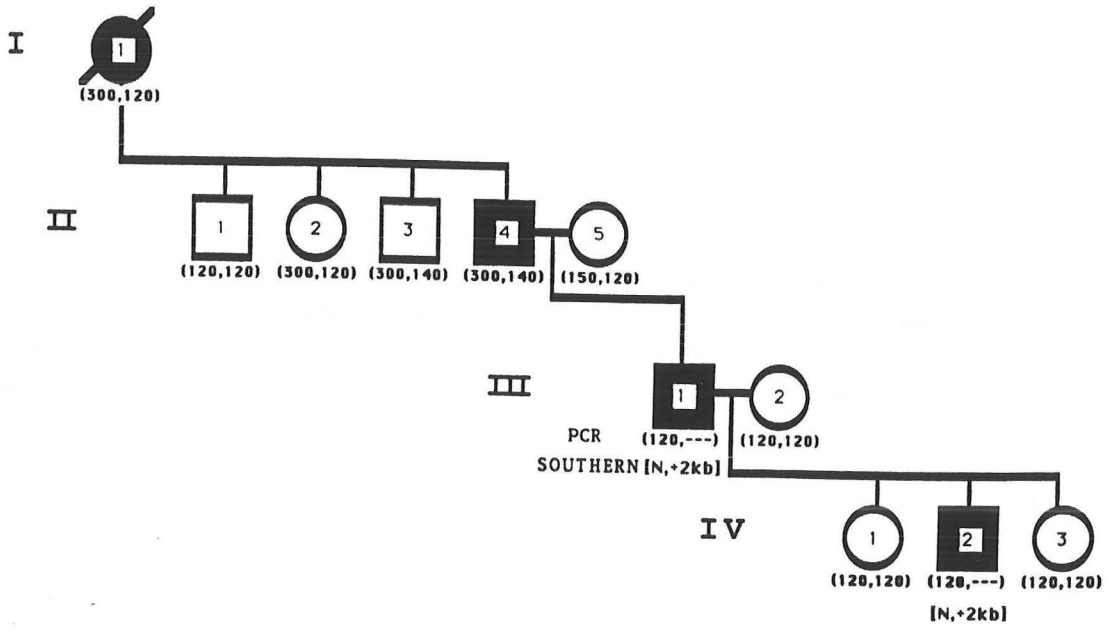

I
1
(300,120)
II
1
2
3
4
5
(120,120)
(300,120)
(300,140)
(300,140)
(150,120)
III
1
2
PCR
SOUTHERN
(120,---)
[N,+2kb]
(120,120)
IV
1
2
3
(120,120)
(120,---)
[N,+2kb]
(120,120)

FIG. 23

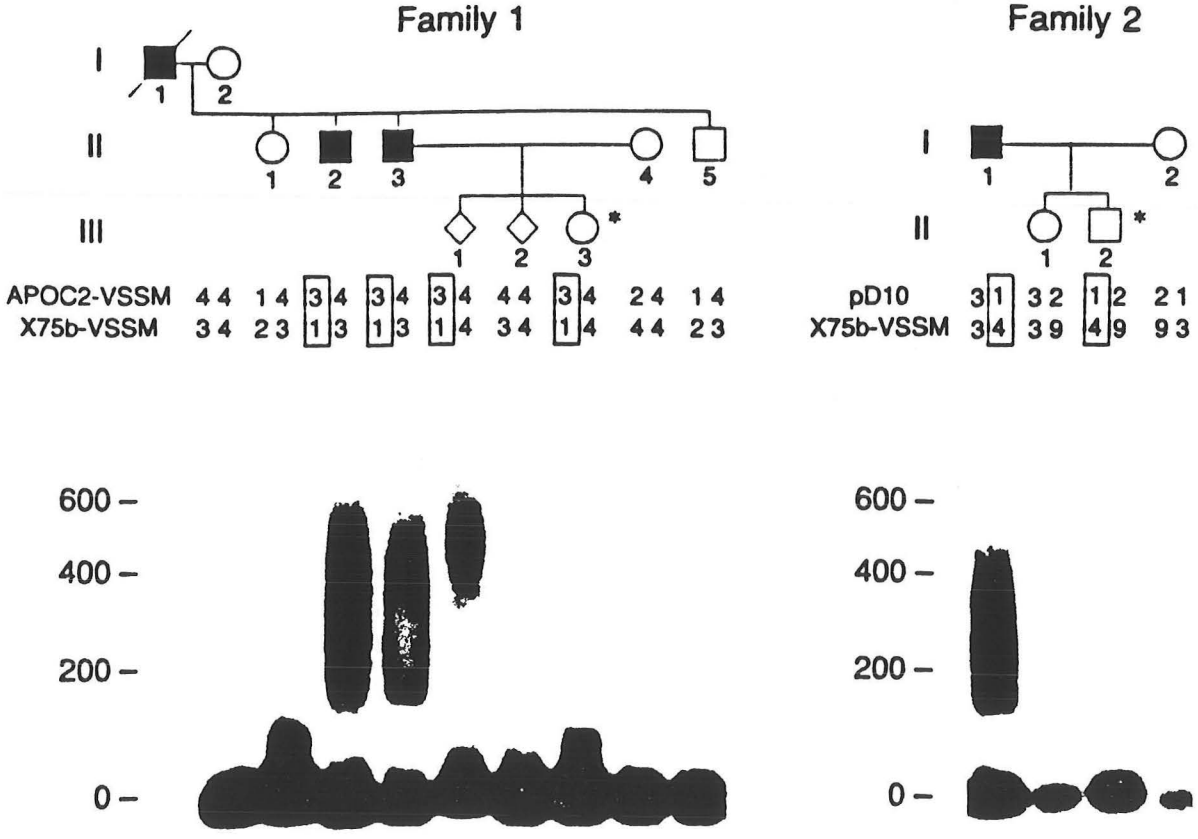

Family 1
Family 2
I
II
III
1
2
3
4
5
*
APOC2-VSSM 4 4 1 4 3 4 3 4 3 4 4 4 3 4 2 4 1 4
X75b-VSSM 3 4 2 3 1 3 1 3 1 4 3 4 1 4 4 4 2 3
pD10 3 1 3 2 1 2 2 1
X75b-VSSM 3 4 3 9 4 9 9 3
600 –
400 –
200 –
0 –

FIG. 24

chromosome truly normal? Once the number of repeats on a chromosome has contracted, will expansion occur again? Does the chromosome somehow remember that it was once expanded? Remarkably, the answer to this question seems to be "yes".

This answer comes from studies of linkage disequilibrium. Using polymorphic markers, one can determine whether all of the chromosomes that give rise to myotonic dystrophy all over the world have a common ancestor. Using these techniques Harley and co-workers have shown that <u>at least</u> 58% of myotonic dystrophy in England and in French Canada can be traced to a single ancestral mutant chromosome (14). This shows that triplet expansion won't occur on any old chromosome 19. There is something special about this chromosome in myotonic dystrophy. There may be a nearby mutation that renders the triplet repeat unstable, allowing it to expand and contract almost at random. This is one theory of the pathogenesis of triplet expansion and I will come back to it at the end of the talk.

I have said a lot about the triplet repeat, and nothing about the gene in which it is located. The groups of Thomas Caskey (10,15) and David Houseman (8) simultaneously determined that the triplet repeat occurs in the 3´ non-coding region of a messenger RNA. The mRNA encodes a protein that has sequence hallmarks of a protein kinase. Fig. 25 shows the alignment of the myotonic dystrophy gene product (designated DM) and several protein kinases (8). The figure shows only the scattered regions of the protein where sequence identities are observed. The related proteins are all known protein kinases. We cannot tell which kind of kinase but the closest animal relative is the cyclic AMP dependent protein kinase. Caskey has named this protein "myotonin-protein kinase" (10).

FIG. 25

Fig. 26 shows a diagram of the mRNA for mytonin protein kinase (16). The complete sequence of the mRNA has not yet been reported. In particular, the sequence does not extend to the 5′ end, nor do we know the sequence of the protein at the amino-terminus. The designation "transmembrane" is only hypothetical. There is a hydrophobic region at this end of the molecule, but it is not yet established that the protein kinase is attached to a membrane. You can see from this figure that the repeats, which Caskey shows in a different phase than the others (i.e.,"GCT") is in the 3′ untranslated region of this mRNA (16).

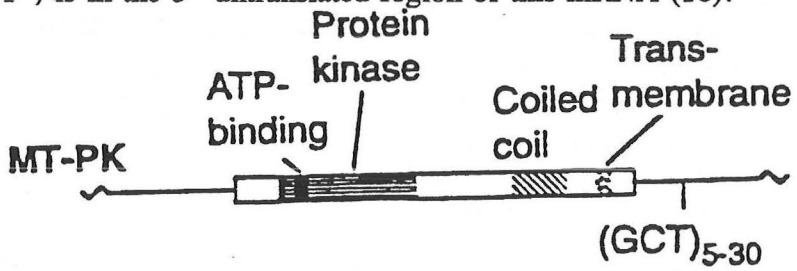


FIG. 26

Where is the mRNA expressed? Northern blots of various tissues are shown in Fig. 27 (17). In this figure the myotonic dystrophy gene is designated DMR-B15. The mRNA

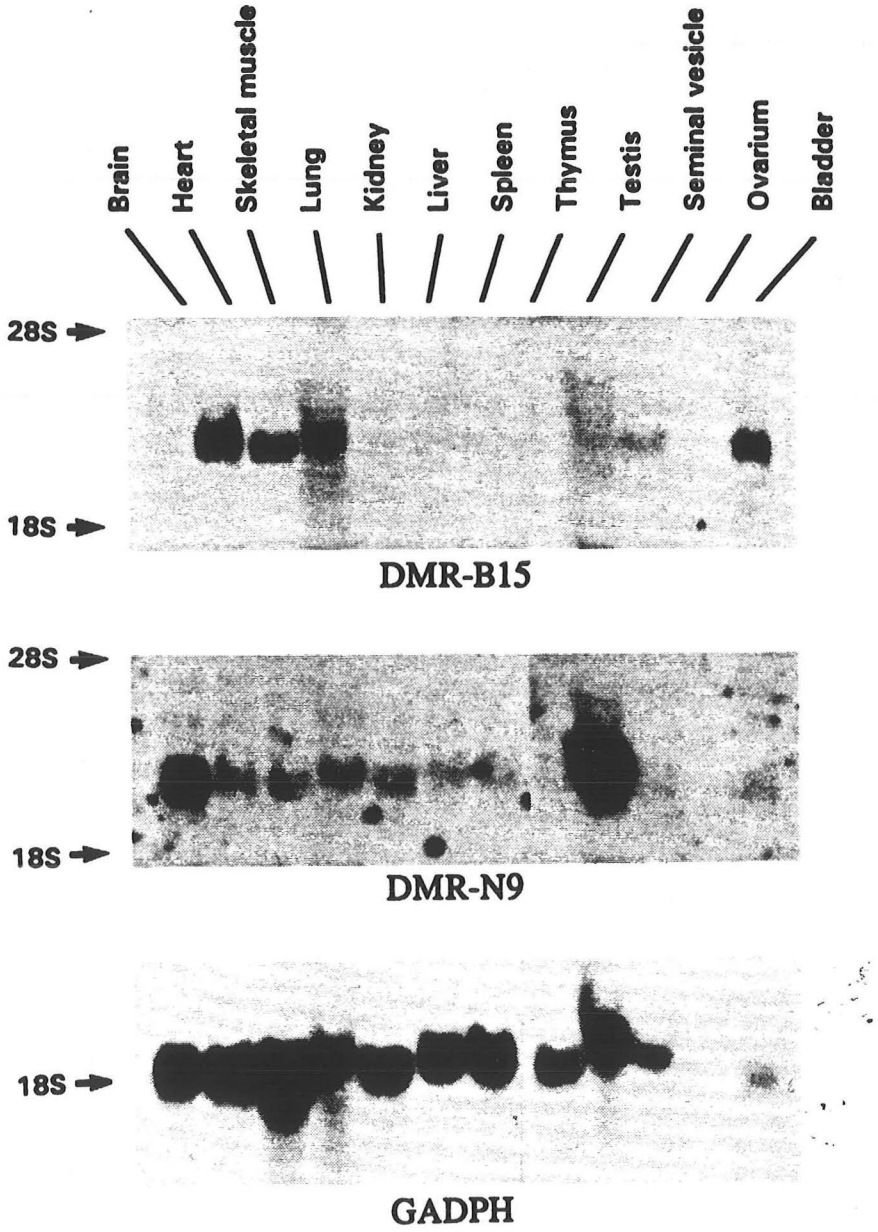


FIG. 27

is expressed highest in the heart, and then in skeletal muscle and lung. The brain shows little expression even though it is a major target of muscular dystrophy, especially the severe congenital form. The Dutch group also identified another gene that is located extremely close to the myotonic dystrophy gene (17). They call this gene DMR-N9. Although the exact transcription units are not known, it seems that the 3´ end of DMR genes N9 and B15 are oriented in the same direction, and the 3´ end of N9 is within 1kb of the 5´ end of B15. The expression of N9 fits more closely with the expected distribution of the myotonic dystrophy clinical picture. It is expressed highest in brain and testis, but also in many other tissues. If the triplet repeat expansion somehow inactivated both genes, then it would affect all of the tissues that are targets in myotonic dystrophy.

We are accustomed to thinking of point mutations as interfering only with the genes in which the mutations occur. It is possible, however, that these triplet mutations exert an affect over a much broader range of the genome. It is difficult to imagine a simple mechanism by which an increase in the number of triplets would produce a disease of progressively increasing severity through the generations. The worst that a huge expansion could do would be to reduce the amount of mRNA by 50% by abolishing expression from the affected chromosome. This 50% deficiency must cause the most severe form of the disease, i.e., the congenital form. The adult onset form must then be attributable to a deficiency of less than 50%. Although this is certainly possible, it is also possible that the triplet expansion prevents the expression of more than one gene on chromosome 19. Moreover, the range of genetic suppression might increase as the number of triplets increases. I'll return to this point later.

The fragile-X syndrome affects 1 in 2,000 males, and 1 in 4,000 females (Fig. 28) (18). It is the most common inherited form of mental retardation (18). Affected individuals have severe mental retardation with IQ averaging 40. They are usually docile but may have violent disruptive behavior requiring institutionalization. They have variable abnormalities in facial features illustrated in this picture of 4 roommates at a mental institution, all of whom have fragile-X. These include coarse facial features with prominence of the ears, forehead, and jaw. The may have joint hyperextensibility and a dilated aortic root suggesting a connective tissue disorder. Nearly all affected adult males have grossly enlarged testes.

Fragile-X syndrome can be singled out from all other causes of mental deficiency by virtue of the abnormal chromosome that is visualized by karyotyping. Fig. 29 shows G-banded metaphase chromosomes from affected individuals (18). In the female, shown on the left one of the X chromosomes shows a break quite close to the end of the long-arm. The break occurs in band XQ 27. The distal fragment is separated slightly from the chromosome. Three examples of single X chromosomes from males with the same appearance are shown.

The Fragile-X site does not exist in life. It is an artifact that is brought out by culturing cells in medium deficient in thymidine or cytidine. Several maneuvers can be used to achieve this deficiency. Pyrimidine deficiency apparently slows the replication of DNA in the region of the mutation, leading to the chromosome break, and the designation "fragile-X". For some time the Fragile-X syndrome has been known to have a peculiar pattern of inheritance, differing

FIG. 28

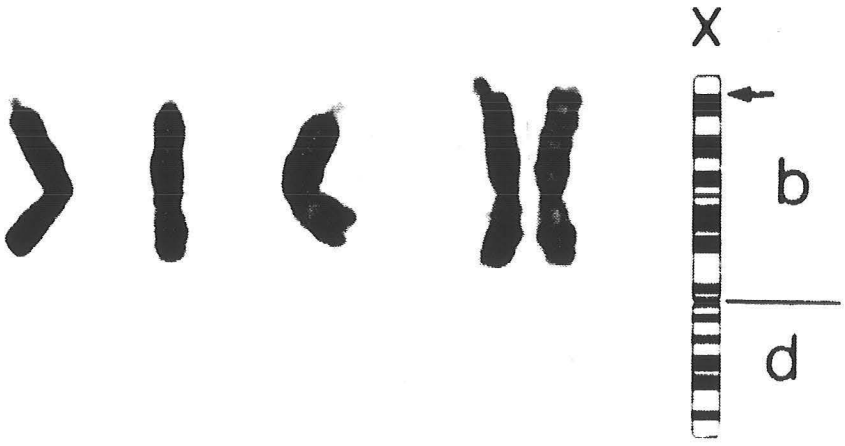


FIG. 29

from all other X-linked disorders. Some of the males have normal intelligence and karyotype even though they transmit the disease genetically. Their daughters are also clinically normal. However, the sons of these daughters have a severe disease. In addition, about 30% of the "carrier" females also manifest the disease, which is unusual for an X-linked trait.

These unusual genetics are illustrated by the idealized family pedigree shown in Fig. 30 (18). The family begins with a female carrier. On average, 9% of the sons and 5% of the daughters of female carriers will have the disease. Subject II-2 has inherited the carrier state, and it appears to have worsened. Her children are more highly affected than her siblings. Forty percent of her sons and 16% of her daughters show the disease. Subject III-2 has the disease. She transmits it to 50% of her sons and 28% of her daughters. In generation II there is a male designated T, which stands for "normal transmitting male". He is intellectually normal, and all of his daughters are clinically normal. However, they are all carriers of the severe trait. Forty percent of their sons and 16% of their daughters will be retarded. The clinically unaffected carriers and the normal transmitting males do not show the Fragile-X histologically, and they are not retarded. Yet they clearly carry a mutation that shows up in later generations. These individuals are said to have a "premutation".

This type of inheritance resembles genetic anticipation as we saw in myotonic dystrophy. It has the unusual feature that severe disease can only be acquired from the mother. As one can imagine, this inheritance pattern caused much confusion among geneticists. It was called the "Sherman Paradox" (19).

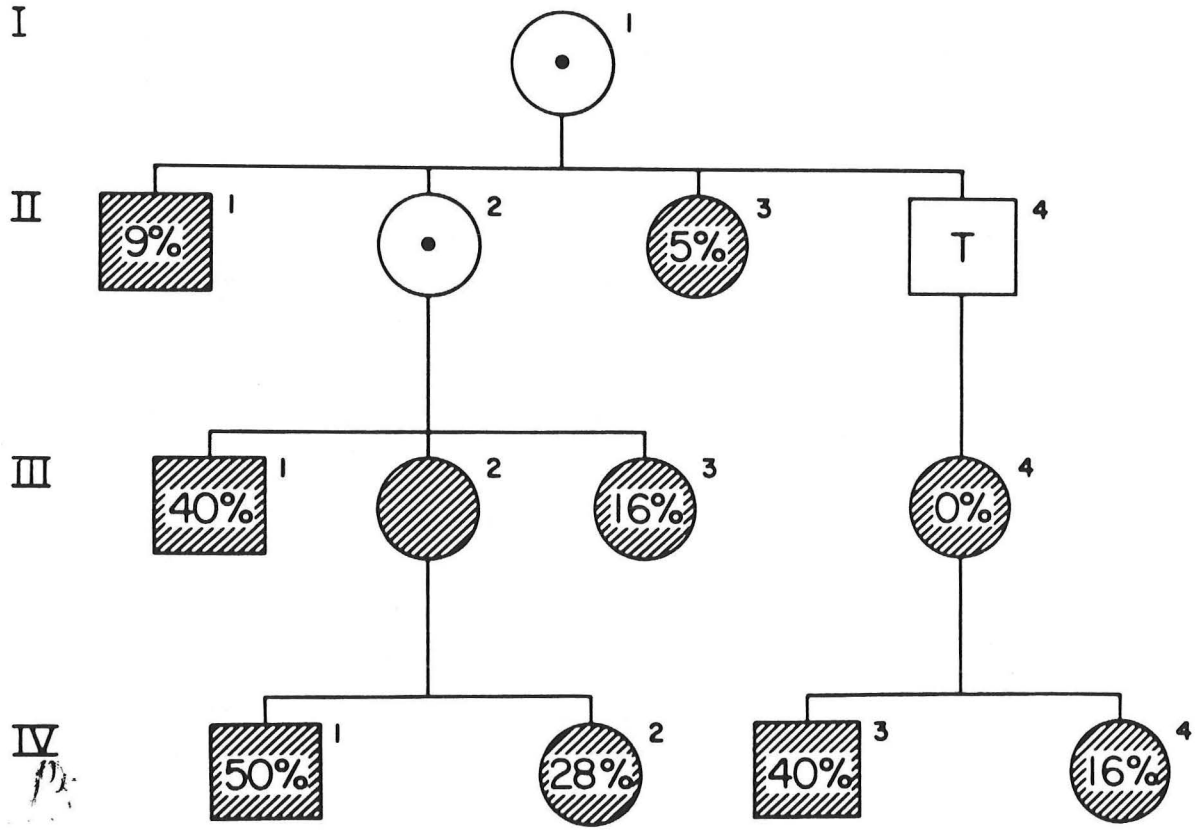


FIG. 30

As shown in Fig. 31, the first step in solving this problem was the demonstration that the defect leading to mental retardation and the site on the X chromosome were closely linked on band-q.27.3 of the X chromosome (18). This region of normal and mutant X chromosomes were isolated by several different groups. One of the groups took the straightforward approach of physically cutting out this region with a needle and cloning it (20a).

In 1991, even before the myotonic dystrophy mutation had been worked out, these groups reported that fragile-X was caused by an expansion of a triplet repeat (20-27). One of the best papers came from Tom Caskey's group at the Baylor Medical School in Houston (26).

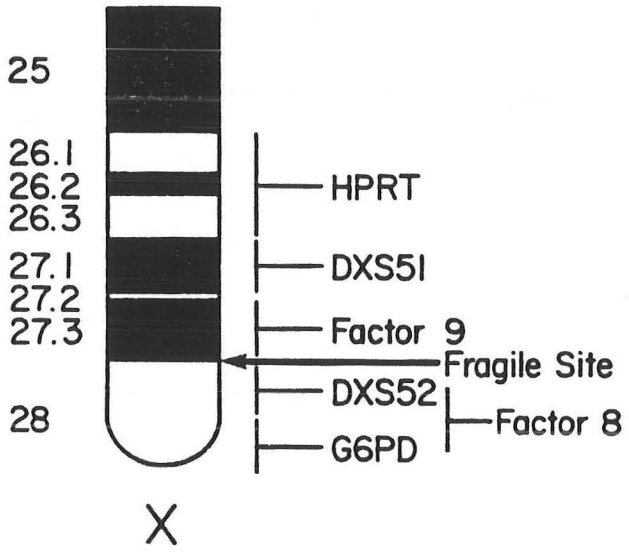


FIG. 31

Fig. 32 shows the sequence of relevant region of the X chromosome with the gene which

```
      CTGCAGAAATGGGCGTTCTGGCCCTCGCGAGGCAGTGCGACCTGTCACCGCCCTTCAGCC
                                                primer g ▶
61    TTCCCGCCCTCCACCAAGCCCGCGCACGCCCGGCCCGCGCGTCTGTCTTTCGACCCGGCA
          PaqI                BssHII          BssHII
121   CCCCGGCCGGTTCCCAGCaGCGCGCATGCgcGCGCTCCCAGGCCACTTGAAGAGAGAGGG
                          SacII                          Sau3AI
181   CGGGGCCGAGGGGCTGAGcCCGCGGGGGGAGGGAACAGCGTTGATCACGTGACGTGGTTT
                                          primer a ▶          ◀ primer h
241   CAGTGTTTACACCCGCAGCGGGCCGGGGGTTCGGCCTCAGTCAGGCGCTCAGCTCCGTTT
                                                          primer c ▶
301   CgGTTTCACTTCCGGTGGAGGGCCGCCTCTGAGCGGGCGGCGGGCCGACGGCGAGCGCGg
        ◀ primer b   end of FMR-1
361   gcggcggcggtgacggaggcgccgctgccaggggcgtgcggcagcgcGGCGGCGGCGGC
                primer d ◀                    XhoI
421   GGCGGCGGCGGCGGCGGCGGCGGCGGCGGCtgaggcctcgagcgCCCGCAGCCCA
                                  NheI          primer e ▶
481   CCTCTCGGGGGCGGGCTCCCGGCGCTAGCAGGGCTGAAGAGAAGATGGAGGAGCTGGTGG
                                                exon boundary    ◀ primer f
541   TGGAAGTGCGGGGCTCCAATGGCGCTTTCTACAAGGTACTTGGCTCTAGGGCAGGCCCCA

601   TCTTCGCCCTTCCTTCCCTCCCTTTTCTTCTTGGTGTCGGCGGGAGGCAGGCCCGGGGCC

661   CTCTTCCCGAGCACCGCGCCTGGGTGCCAGGGCACGCTCGGCGGGATGTTGTTgGGAGGG

721   AAGGACTGGACTTGGGGCCTGTTGGAAGCCCCTCTCCGACTCCGAGAGGCCCTAGCGCCT

781   ATCGAAATGAGAGACCAGCGAGGAGAGGGTTCTCTTTCGGCGCCGAGcCCCGCCGGGGTG

841   AGCTGGGGATGGGCGAGGGCCGGCGGCAGGTACTAGAGCCGGGCGGGAAGGGCCGAAATC
                                                                BamHI
901   GGCGCTAAGTGACGGCGATGGCTTATTCCCCCTTTCCTAAACATCATCTCCCAgCGGGAT

961   CCGGGCCTGTCGTGTGGGTAGTTGTGGAGGAGCGGGGGGCGCTTCAGCCGGGCCgCCTCC

1021  TGCAG
```

FIG. 32

is named FMR-1 (Familial Mental Retardation-1) (26). The sequence contains a triplet repeat (CCG). PCR was used to show that the number of CCG repeats varies in the population.

The upper panel of Fig. 33 shows the frequency of repeats among normal people (26). The most frequent number was 29, and only one normal subject had more than 50 repeats. The bottom panel of Fig. 33 shows the number of repeats in carriers who have a "premutation" for fragile-X. In each case the number of repeats is above 50, ranging from 51-250.

The inheritance of the unstable repeat in a family with fragile-X is shown in Fig. 34 (26). The grandmother has a premutation allele with 73 CCG repeats. When transmitted to her daughter this has expanded to 83 repeats. The daughter had two sons with fragile-X syndrome. In both of them the gene had undergone a dramatic expansion with thousands of CCG repeats. Because of limitations of the PCR technique such a large band could not even be visualized on the gel. Other methods confirmed that this disappearance is caused by a huge expansion.

The Caskey group, (26,27) and several others, (20-25) have studied many patients with full-blown Fragile-X syndrome and they always observed massive expansion of the triplet repeat, just as in myotonic dystrophy. The major difference is that in myotonic dystrophy the triplet expansion can occur during passage from either the father or the mother. In fragile-X syndrome, massive expansion occurs only when the gene is transmitted by the mother.

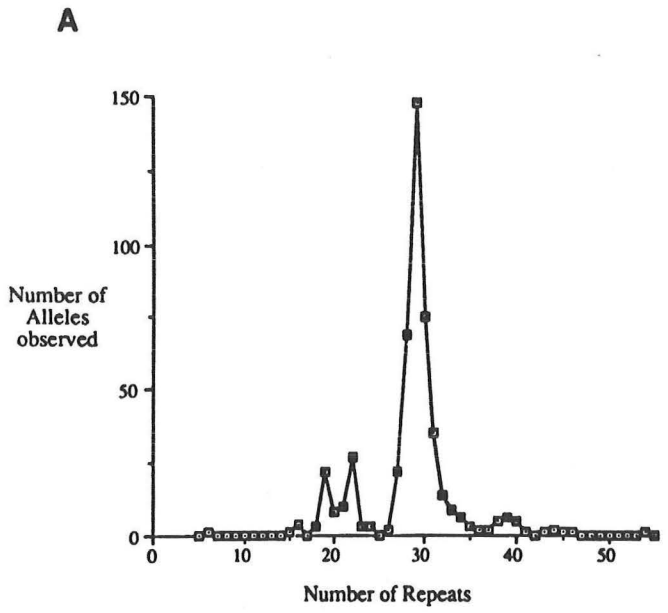
As shown in Fig. 35 the chance of undergoing a massive expansion is proportional to the number of triplet repeats in the mother's genes (26). When the number of CCG repeats in the mother was above 90, the allele underwent expansion at every meiosis. This finding offers a complete explanation of the Sherman Paradox.

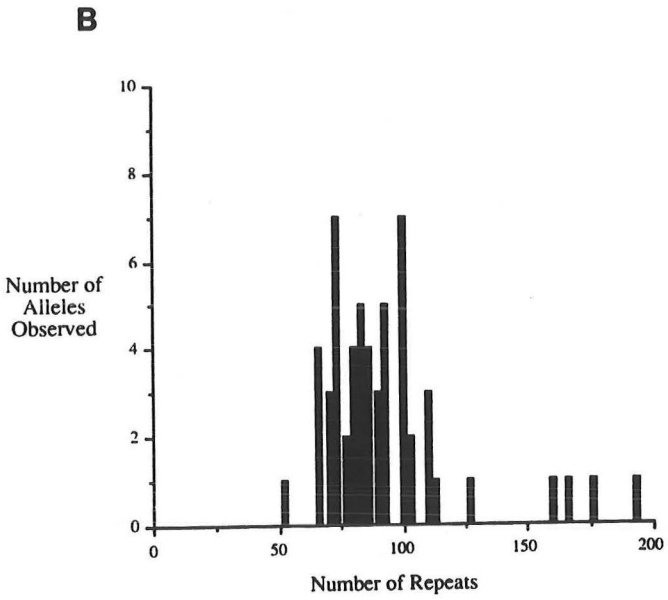
Fig. 36 shows an idealized pedigree relating the number of triplet repeats to the Sherman Paradox (26). The grey symbols designate unaffected carrier women with "premutations". The risk of expansion to the full mutation increases progressively as the number of repeats in the "premutation" carriers increases.

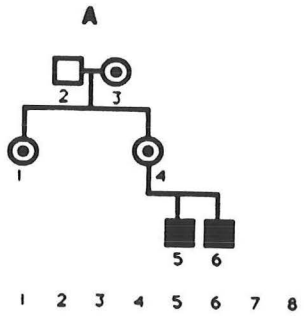
How does the fragile-X mutation cause disease? We know that the triplet repeat is the exact site of the break that occurs in thymidine-deficient media. However, this disruption is not believed to occur *in vivo*. Chromosome disruption does not cause the disease. Rather, the disease is attributed to the reduced expression of the FMR-1 gene which is the gene in which the triplet expansion occurs (28).

The entire cDNA for the FMR-1 protein has not been cloned, and the sequence of the FMR-1 protein has not been elucidated completely. The information now available indicates no resemblance to any known protein. The CCG repeat was initially thought (mistakenly)(27) to occur within the coding region of the gene, encoding a string of arginine residues. This idea has been abandoned. Workers now feel that this repeat is in the 5′ untranslated region of the mRNA encoding the FMR-1 protein.

FIG. 33


A
150
100
50
0
Number of Alleles observed
0
10
20
30
40
50
Number of Repeats


B
10
8
6
4
2
0
Number of Alleles Observed
0
50
100
150
200
Number of Repeats

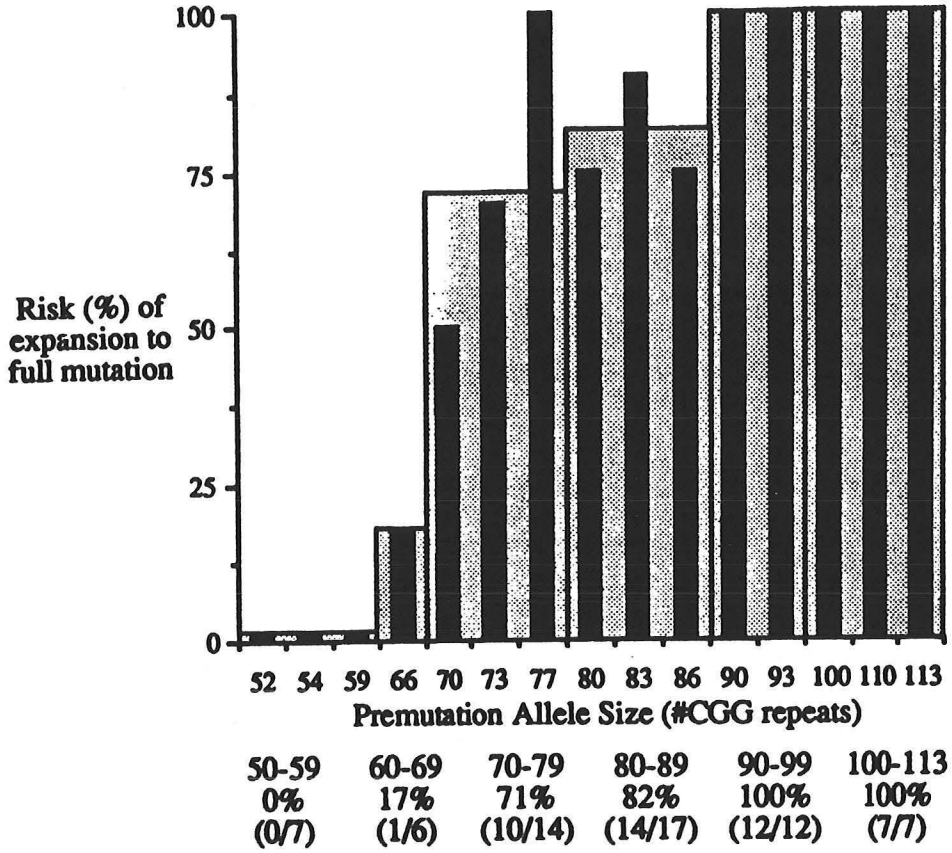

A
2
3
1
4
5
6
1 2 3 4 5 6 7 8


83 repeats
73 repeats


29 repeats

FIG. 34

FIG. 35


Risk (%) of expansion to full mutation
100
75
50
25
0
52 54 59 66 70 73 77 80 83 86 90 93 100 110 113
Premutation Allele Size (#CGG repeats)
50-59 0% (0/7)
60-69 17% (1/6)
70-79 71% (10/14)
80-89 82% (14/17)
90-99 100% (12/12)
100-113 100% (7/7)

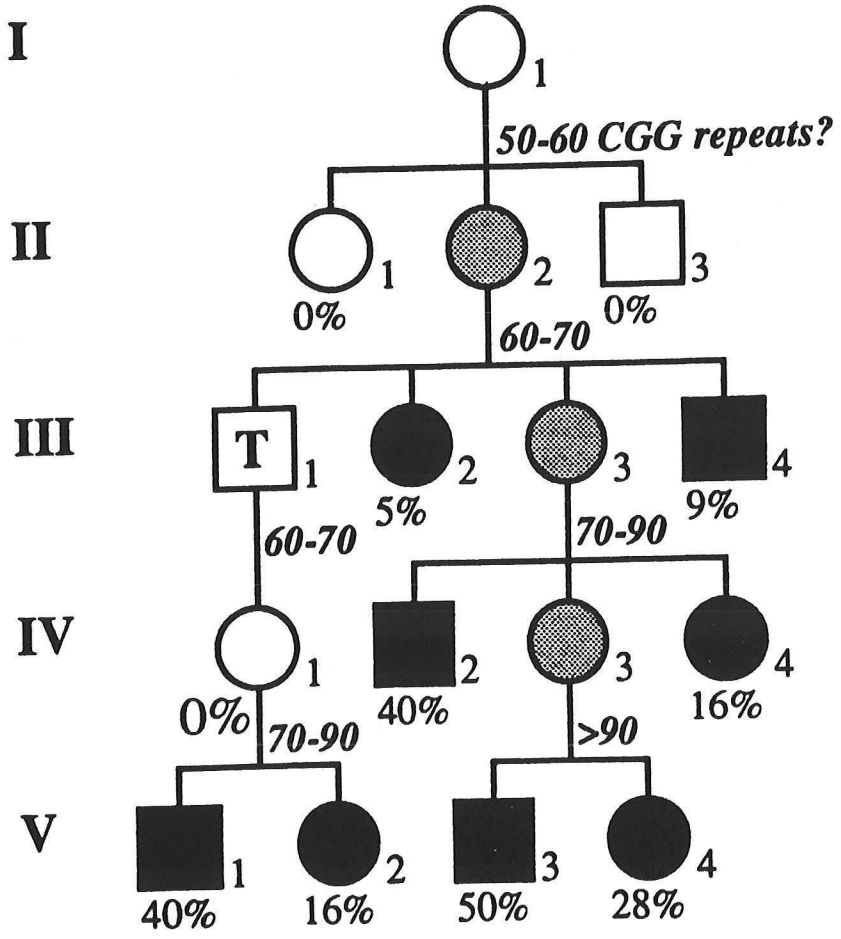

I
II
III
IV
V
1
50-60 CGG repeats?
1 0%
2
3 0%
60-70
T 1
2 5%
3
4 9%
60-70
70-90
1 0%
70-90
2 40%
3
>90
4 16%
1 40%
2 16%
3 50%
4 28%

FIG. 36

Triplet expansion prevents the expression of the FMR-1 mRNA. Caskey's group failed to detect the mRNA in lymphoblasts from 16 out of 20 boys with Fragile-X syndrome (28). In cells from 4 other affected boys the expression was normal. The authors explained this by stating that in tissue culture the number of repeats had contracted, thereby allowing expression of the gene. This has not yet been proven.

The reason for the lack of expression appears to be methylation occurring in the FMR-1 gene that is induced by the presence of the CCG repeat (29). Throughout the genome there are clusters of CG-dinucleotides located adjacent to genes. The C frequently undergoes methylation to form 5 methylcytosine.(29) Methylation inactivates the gene, and it is highly controlled by developmental factors. The triplet repeat in the fragile-X syndrome differs from the repeats in the other two diseases: it contains a CG-dinucleotide (CCG). Studies have shown that this dinucleotide is methylated in affected individuals, as are nearby clusters of CG-dinucleotides (29). This methylation appears to be induced by the presence of the CCG triplet expansion.

Methylation of CG-dinucleotides is particularly important in the case of the X chromosome (29). In female cells only one copy of the X chromosome is active. The other copy is highly methylated and inactive. In females carrying the full mutation at the fragile-X locus, nearby CG dinucleotides have been shown to be methylated (21). Thus, the CCG repeat may induce the selective methylation of one region of the X chromosome.

Our understanding of Fragile-X was increased markedly last month when a paper from Belgium reported a boy with Fragile-X who had a point mutation in the FMR-1 gene.(31) This boy had the complete Fragile-X syndrom including severe mental retardation and truly impressive macro-orchidism. Karyotyping showed no fragile site and he had only 25 CCG repeats, which is normal. The FMR-1 gene had a point mutation that changed an isoleucine to arginine, This mutation was not observed in 130 normal X chromosomes. The patient had no family history of mental retardation, and his mutation was shown to be a new mutation. This finding provides conclusive evidence that the entire clinical picture of Fragile-X syndrome is caused by defective function of the FMR-1 protein. There is no need to postulate involvement of any other area of the X chromosome.

The final disease traced to triplet expansion is X-linked spinal and muscular atrophy, also known as Kennedy's disease (Fig. 37). It is an adult onset motor neuropathy with signs of

X-Linked Spinal And Bulbar Muscular Atrophy (Kennedy's Disease)

Only males affected
Onset age 20-45
Progressive weakness with fasciculations
Gynecomastia, reduced fertility, testicular atrophy

FIG. 37

androgen deficiency. Linkage studies localized the mutation to the same band on the X chromosome as the androgen receptor (30). Using the receptor cDNA as a probe a team at the University of Pennsylvania traced the defect to an expansion of a triplet repeat within the coding region of the androgen receptor gene (30).

Fig. 38 compares the mutation in Kennedy's disease with the other two triplet repeat diseases (16). In Kennedy's Disease the triplet repeat CAG, is found in the coding region of the gene for the androgen receptor (AR), where it encodes a stretch of glutamines (30). This sequence is polymorphic in the normal population, with an average of 21 repeats and a range of 17 to 26. In Kennedy's disease the number is roughly doubled, ranging from 40 to 52. There is a slight tendency to increase in size in subsequent generations, but there is no dramatic expansion to hundreds of repeats as occurs in the other two diseases (30,32).

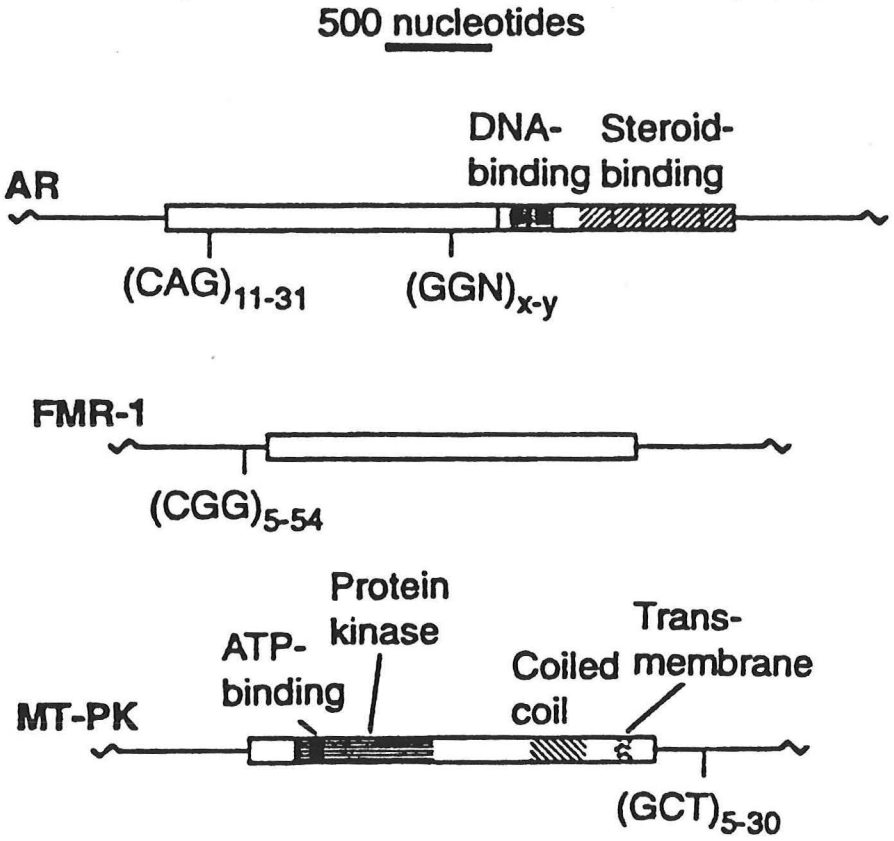


FIG. 38

The increased glutamines occur in a region of the androgen receptor that is believed to activate gene transcription. So far, it is not clear whether the extra glutamines actually affect the function of the receptor protein. Individuals who lack the androgen receptor have complete testicular feminization. They fail to develop male genitalia, but they have no neurologic abnormality. This means that the triplet expansion in Kennedy's disease does more than inactivate the androgen receptor. It might endow this receptor with new properties, thereby causing a dominant effect on neurons. It also seems possible that the triplet expansion inactivates more than the androgen receptor gene - it may somehow interfere with the expression of other nearby genes. I'll return to this idea in a moment.

Sutherland has reported strong evidence for founder effects in the Fragile-X syndrome (36). It appears that these unstable chromosomes are descended from a small number of ancestral mutant chromosomes. This resembles the situation in myotonic dystrophy. The implications will be discussed in a moment.

Fig. 38 also shows the locations of the triplet repeats in Fragile-X syndrome (FMR-1) and in myotonic dystrophy (MT-PK) (16). Recall again that these repeats are not in the coding regions but in the transcribed flanking regions of these genes.

How does triplet expansion occur? We don't know precisely, but the general feeling is that it occurs through slipped mispairing (33,34,35). During DNA replication the complementary strands align with one another. Repeat sequences can align off-center, leading to duplication of the two overhanging ends.

Why should these triplets expand? Two theories have been proposed by Sutherland, and these are shown in Fig. 39 (35). Situation A envisions that the unstable chromosomes have a

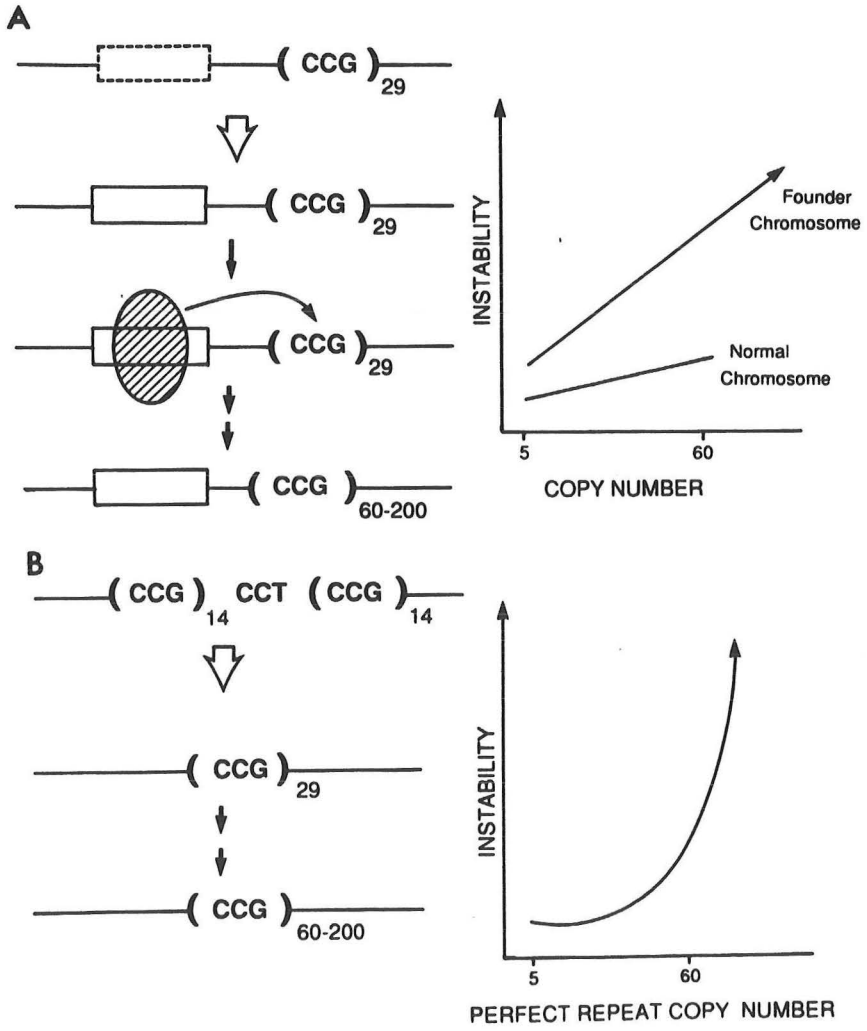


FIG. 39

mutation near the triplet repeat that destabilizes the repeat, leading to expansion.
Situation B envisions that the rate of expansion is geometrically proportional to the number of repeats. Normally when repeats expand they undergo point mutations that destroy the perfect repeat sequence. These interruptions prevent further expansion since slipped base pairing can no longer occur. However, on rare occasions there is a perfect expansion and this creates the possibility of further slipped mispairing and a dramatic expansion.

I strongly favor the first explanation (Panel A) because of the founder chromosomes. The triplet expansion that gave rise to the unstable chromosomes of myotonic dystrophy and Fragile-X are very rare events. They only happened a few times in human history. This strongly suggests a highly specific mutation. If unstable chromosomes could be generated whenever perfect expansion occurs this event would have occurred much more frequently, simply by chance. I believe that nature has a way of preventing slipped mispairing, and this protective mechanism has been abolished by a nearby mutation on the founder chromosome.

If a founder mutation exists it has implications for genetic counseling. Recall the families with myotonic dystrophy in which the number of repeats contracted. The descendants of these individuals may show triplet expansion because their chromosomes still harbor the founder mutation.

How do the triplet expansions cause disease? Fig.40 compares all 3 diseases, looking for common features. All 3 diseases have triplet expansions. They involve different chromosomes and different genes. All are in transcribed genes, but the spinal atrophy repeat is the only one that affects the structure of a protein.

DISEASES CAUSED BY EXPANDED TRIPLET REPEATS

	MYOTONIC DYSTROPHY	FRAGILE-X	SP. MUSCULAR ATROPHY
Triplet	**CAG**	**CCG**	**AGC**
Chromosome	**19**	**X**	**X**
Gene	**Protein Kinase**	**FMR-1**	**Androgen Receptor**
Location	**3′ UTR**	**5′ UTR**	**Coding (Glutamine)**
Anticipation	**Yes**	**Yes**	**No**
Meiotic Expansion	**Either Parent**	**Maternal**	**Either (Limited)**
Mitotic Expansion	**Yes**	**Yes**	**?**
Founder Chromosome	**Yes**	**Yes**	**No (?)**

FIG. 40

These mutations may simply inactivate the genes in which they occur. This is apparently the case in Fragile-X in which a point mutation in the FMR-1 gene can produce the whole syndrome, as I discussed above.

There is an alternative explanation and its justification is shown in Fig.41. The figure shows the triplet repeats in each disease. In each case I have shown only one strand of the DNA, and I have chosen the strand to illustrate the fundamental uniformity in these sequences. As shown by the boxes each of these sequences conforms to a single motif: "GC Pyrimidine GC Pyrimidine". I can't believe that this happened by chance.

Expanded Triplet Repeats

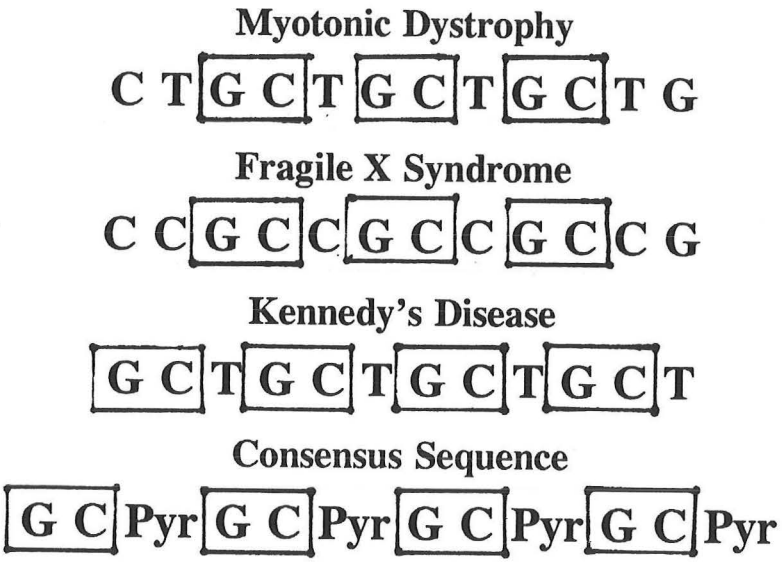


FIG. 41

I would like to suggest that GCPyr repeats are inherently destabilizing. They may distort the structure of DNA or they may distort the normal arrangement of nucleosomes. They certainly prone to sudden expansion. They may also interfere with gene expression in a widespread fashion.

If they decrease the expression of nearby genes the expanded repeats would produce an effect like a small chromosomal deletion. Mitotic expansion accentuates the effect so that somatic cells are affected to progressively worsening degrees. This may help to explain why myotonic dystrophy and spinal muscular atrophy are late-onset diseases.

Finally, I wish to turn to other diseases that show genetic anticipation. As shown in Fig. 3 Penrose identified six diseases that fit this inheritance pattern, in addition to myotonic dystrophy (1). Huntington's disease is a particularly well-documented example.

Juvenile Huntington's Disease, like congenital myotinic dystrophy, appears to be the end-product of anticipation accumulating after several generations (37). Another example is Type II diabetes mellitus. It is second only to myotonic dystrophy in Penrose's list. We have all seen young people with Type II diabetes whose parents have classic late-onset disease. This seems particularly common among African-Americans. I believe that one form of Type II diabetes will be traced eventually to a triplet expansion in a gene regulating energy metabolism.

The lessons learned over the past 2 years should alert all of us to the phenomenon of genetic anticipation. I believe that many more examples will soon be brought to the fore.

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