Fragile sites on human chromosomes- a personal odyssey

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Preamble

The study of fragile sites on human chromosomes has preoccupied me for more than two decades of my professional life. During this period others in the Department have contributed to these studies at levels ranging from purely technical assistance to major intellectual input and the application of essential technologies and strategies.

This narrative endeavours to create a record of progress made in this area and to acknowledge the contributions made by colleagues, It is not a review in the usual scientific sense and thus does not acknowledge all the contributions from other groups.

The Beginning

I commenced work at the Adelaide Children's Hospital (ACH) on the 2nd of January 1975. I was a reasonably experienced cytogeneticist and had just obtained my PhD in Edinburgh on the properties of amniotic fluid cells in tissue culture, with an emphasis on prenatal diagnosis of genetic disease. Because I had previously worked in a cytogenetics laboratory in Melbourne which served the medical practitioners assessing and managing the intellectually handicapped, and because the main disorder that could be prenatally diagnosed was Down syndrome I had developed, and maintained, a major interest in the genetics of mental retardation.

On arrival at ACH I was told by Rod Carter who had recruited me that half the resources at my disposal (staff of four) in the Cytogenetics Unit within his Department of Histopathology were for diagnostic cytogenetics and the remainder were for research. What to do? I was unsure. This was not at that time an appropriate environment to pursue my prenatal diagnostic interests. The records of the Unit contained information on a number of individuals, then mostly resident in a residential centre for the mentally retarded, who had been found to have fragile sites on their autosomes. Fragile sites had been described in the literature, but little was known about them. I decided to restudy the patients with fragile sites as this would give me a publication and give me time to think of a significant area of research.

I did restudy the patients but was not able to find the fragile sites which had been previously recorded. I had a problem to study but was it a significant one? Late in 1975 I visited my old laboratory in Melbourne and the Cytogeneticist who had replaced me there, Jill Harvey, showed me some families she had been working on who had fragile X syndrome. [These were published in 1977 and this paper greatly helped generate interest in the fragile X (40)]. I thought that the fragile X was probably akin to the autosomal fragile sites that I had begun to try and look at. I was keen to find some fragile X families to work on but knew I would have to sort out my technology first. At that time I recalled the original fragile (then called marker) X family described by Lubs in 1969(43). I had read his paper at that time of publication in detail and with fascination.

Back in Adelaide I tried to work out what I needed to do to see the fragile sites. After several fruitless experiments with antibiotics added to culture medium I decided to replicate entirely the culture conditions I knew well that were still used in the Melbourne laboratory. This involved obtaining culture medium TC199 as the Adelaide laboratory (along with many other laboratories) had changed to more modern culture media such as Ham's F10 and RPMI1640. The fragile sites I was studying reappeared! By study of patients with apparent X-linked mental retardation I also rapidly found some fragile X families. I announced the finding of the need to use TC199 during the discussion of a presentation by Malcolm Ferguson-Smith at the ACH Centenary Scientific Meeting(39) in August 1976 and later that year at a presentation to the 182nd Meeting of the Genetical Society in London. Those were times when unpublished findings were openly discussed in scientific meetings. This work was subsequently published as a letter to the *New England Journal of Medicine* (1) and in *Science*(2) (after being rejected by *Nature*).

What's in TC199?

Thoughts of other projects were on hold. I now had a way of seeing fragile sites and one of them, the fragile X, was of some relevance to the genetics of mental retardation. What was it about TC199 that allowed the fragile sites to be seen in lymphocyte culture? This medium contains many more components than newer culture media and it seemed reasonable to assume that one of these was responsible. It is a major undertaking to produce a series of culture media, each with a different component omitted. I did some experiments along these lines with the help of Andrew MacGregor at Commonwealth Serum Laboratories but nothing eventuated.

With some sense of frustration, I regularly reviewed the recipe for TC199 in comparison with other media. One day I noticed that TC199 contained much less folic acid than the others. At last an easy experiment. I added folic acid to TC199 and the fragile sites disappeared. I still remember the day I looked at the slides I had prepared.

Discussing this result with Andrew MacGregor led me to look at thymidine, as a result of some now illogical thoughts (of mine) about HAT medium. Thymidine added to TC199 also caused fragile sites to disappear. I published this work and a lot of experimental data on concentrations, times of effect, medium pH, cell types and the effects of inhibitors of folate metabolism in two back to back papers in the American Journal of Human Genetics in 1979(3,4). A lot of chromosome harvesting and microscopy that produced data in these papers was well performed by Lynne Day, Helen Eyre, Trudy Hocking and Erica Woollatt, the total staff of the Cytogenetics Unit at that time. These papers had been reviewed by Fred Hecht, who with his wife Barbara wrote an accompanying editorial (41). This began a long friendship and association. Fred stimulated me to write a book (14) which we co-authored during a short sabbatical I spent at his Institute in Phoenix, Arizona in 1983.

As the fragile X was slowly being found in other laboratories the clinical aspects were beginning to emerge. Gill Turner's work in NSW on X-linked mental retardation was gaining considerable attention. X-linked mental retardation with macroorchidism was described. A

connection between the two was made when I enlisted the help of Histopathology Registrar, Peter Ashforth (later a child psychiatrist) to measure the testes of some fragile X males I had identified(5). Controversy raged in the literature for a while about whether macroorchidism was indeed a feature of fragile X syndrome but the 'measurers' triumphed over the 'visual inspectors' and the now long list of clinical features associated with fragile X syndrome emerged.

Peter Jacky arrived in the laboratory from Oregon in 1981 with a claim that he could induce the fragile X in fibroblasts(42). We were never able to repeat this reliably but were able to use the approach to demonstrate the fragile X in cultured amniotic fluid cells and thus enable prenatal diagnosis of fragile X (6). Later, we were able to use molecular technology to demonstrate that this approach could reliably prenatally diagnose fragile X(26).

Also at this time Berndt Beek spent a short sabbatical with us and this collaboration resulted in another *Science* paper(10) showing that the conditions of tissue culture that induced fragile sites increased the frequency of micronuclei. Culture medium composition was thus important in mutagenesis studies with micronucleus formation as an assay.

Liz Baker, while working with me on an unrelated project involving SCE discovered the BrdU-requiring fragile site at 10q25 (FRA10B)(7). She assisted greatly, along with Peter Jacky, in demonstrating the differences and similarities between FRA10B and the fragile sites (FRAI6B and FRA17A) inducible by Distamycin A (as well as BrdU). Liz had been the foundation member of the Cytogenetics Unit from 1965-70 and returned in 1978 to work as my research assistant on other projects but soon was spending most of her time looking at fragile sites and did much of the work we jointly published since 1980.

Genetic Markers

John Mulley joined the group in 1978. I had, following a visit to Malcolm Ferguson-Smith's Duncan Guthrie Institute in Glasgow, wanted to establish a genetic marker laboratory, with a view to being able to perform prenatal diagnosis by linkage. At that time only red cell and protein polymorphisms were available and John had experience with these. We had not anticipated the discovery of DNA polymorphisms but found ourselves well placed to exploit these.

Initially we showed that there appeared to be an excess of carriers of mildly deficient alpha-1 antitrypsin (PI) genotypes amongst the most ancestral individuals we could identify in families with the folate sensitive, but not other, rare fragile sites(12). We have never repeated this study, but it may be worth revisiting in the light of potential trans-acting factors being involved in dynamic mutations.

We went on to show, using fragile site family material, that fragile sites did not appear to affect meiotic recombination that could be detected by any changes in genetic distances around fragile site loci in those with and without fragile sites(8,11).

Molecular Genetics

By 1981 I had formed the view that we would learn little more about the fundamental nature of fragile sites unless we could clone them. I had thought that by understanding the molecular mechanisms of fragile site formation it would have been possible to modify tissue culture conditions to allow fragile sites to be seen in close to 100% of cells, thus greatly facilitating their cytogenetic detection. This has not happened and is now not necessary. I established, slowly, molecular genetics within the Department by employing a series of young post-docs with molecular genetics experience and taking on PhD students with a grounding in molecular biology. This was a time when it was difficult to find scientists with an in-depth knowledge of molecular genetics and it was not until Val Hyland was recruited from Ireland in 1984 that we had technology well enough established to begin generating data.

In 1983 David Callen joined the group. David had skills in somatic cell genetics and we decided that we would positionally clone the fragile sites on chromosome 16. Positional cloning was a concept then rather than a technology. In conjunction with the molecular biology skills of Val Hyland and Antonio Fratini (a PhD student) David set out to construct a somatic cell hybrid panel to map chromosome 16(38). We chose chromosome 16 because it had at least three (now four) different types of fragile site and the *APRT* gene which could be selected for in somatic cell hybrids. (This somatic cell hybrid work went on to be very successful and lead the group into its involvement in the human genome project). We had decided initially not to tackle cloning the fragile X; we thought it might be too competitive. However when the complementary somatic cell hybrids CY2/CY3 were made by David Callen we changed our views on this. CY3 contained most of chromosome 16 and the end of the long arm of the X chromosome. Using this hybrid Val Hyland and Kirk Fernandez (a Research Assistant) produced and mapped the VK series of clones to chromosomes 16 and X(19), (We did not know at the time that VK16 was within the *FMR1* gene and VK21 was within the *FMR2* gene).

From about mid 1978, all our diagnostic cytogenetics was performed in a way that allowed folate-sensitive fragile sites to be detected, and from mid-1980 we also tested all diagnostic referrals for BrdU- inducible fragile sites. We built up a collection of fragile site families, including fragile X syndrome families, that is still unrivalled, and these families have been and remain a unique resource for much of the fragile site research that we have performed. The fragile X families allowed us to generate data that enabled virtually unequivocal diagnosis of fragile X carriers within families who did not express the fragile site cytogenetically by computerised risk analysis before the fragile X was cloned(17).

Using our fragile site family material I collaborated with Stephanie Sherman (a genetic epidemiologist working with Newton Morton and Pat Jacobs, then in Hawaii) on a segregation analysis of fragile sites. Stephanie had initially performed a segregation analysis on a group of fragile X families(45), which was confirmed on an extended group of families(13), with remarkable results. The fragile X had the highest mutation rate of any

locus that had been studied in the human genome, males could transmit (while asymptomatic) an X-linked recessive gene, the risk of female carriers of the fragile X having a child with the syndrome depended upon their place within the pedigree (the Sherman paradox) and whether they were mildly affected with the disorder themselves.

We showed(16) that the autosomal folate sensitive fragile sites were also unusual in that penetrance when transmitted by females was approximately 100%, but when transmitted by males was only 50%. This offered a partial explanation for the puzzling observation that the carrier parent of new ascertainments with autosomal fragile sites was almost always the mother. Fragile sites had rapidly gained the aura of a unique genetic phenomenon.

In 1984 Liz Baker eventually convinced me that high levels of thymidine, used in our diagnostic laboratory to synchronise lymphocyte cultures, were also inducing the folate sensitive fragile sites. We investigated this systematically and found that there was a U-shaped dose response of these fragile sites to thymidine concentration, but not to BrdU(15). I still do not know why high concentrations of BrdU do not induce these fragile sites.

Concurrently Ruth Simmers joined us as a PhD student (1984-1987) and developed isotopic in situ hybridisation to chromosomes for mapping probes primarily as an adjunct to our positional cloning approaches which were ongoing. Ruth located very precisely some of the few genes then known to be on chromosome 16. During this time others(48) had suggested that fragile sites could predispose to cancer and that the breakpoints seen in non-random chromosome rearrangements in malignant disease were likely to be at fragile sites. Ruth showed that in a couple of strongly asserted examples of this, that the fragile sites and the malignancy breakpoints did not coincide(18). As Ruth was finishing her PhD Liz Baker took on the in situ hybridisation technology, eventually converting to fluorescent rather than isotopic labelling of probes. (I was personally delighted when Liz obtained her MMedSc in 1997, having been accepted as a graduate student by the University of Adelaide on the basis of her published work, without having a primary degree - a rare achievement).

In 1987 Graeme Suthers, a paediatrician who would eventually become a clinical geneticist in the Hospital, came to the group as a PhD student. His project involved the detailed linkage (20) and physical mapping(21) of the region of the X chromosome around the fragile site. Graeme organised an International consortium to put together a somatic cell hybrid panel to physically map probes within the region, and to determine linkage relationships. This work was very successful, it put to rest claims made by others that the presence of the fragile site disturbed genetic distances(23) and laid the foundation for the positional cloning of the fragile X.

Cloning the fragile X

The final phases of our isolation of the fragile X DNA sequences began with the concerted effort to build upon Graeme Suthers' mapping studies. This was coordinated by Rob Richards, who had joined the Department in 1989 and included post-docs Melanie Pritchard, Eric Kremer and Michael Lynch and a young Chinese clinical geneticist, Sui Yu, who had joined us as a PhD student. We initially collaborated and then competed with some of the

other groups working in this area, David Schlessinger provided us with a YAC containing the fragile site, Jean-Louis Mandel contributed some probes in the area, Steve Warren gave us access to somatic cell hybrids with breaks at the fragile site. The paper on the cloning of the fragile X appeared in *Science* in 1991(25), in the same issue that Mandel's group published their findings which were similar to ours (44). Shortly afterwards the other major groups (a consortium led by Tom Caskey in Baylor, Steve Warren in Atlanta and Ben Oostra in Holland) published the finding of the *FMR1* gene in *Cell*. 1991 was a great year for fragile site aficionados. Although there were many competing groups we had won the race to clone the fragile X and held the patent on the use of the fragile site for diagnosis.

Trinucleotide repeats

The cloning of the fragile X, resulted in the discovery of a novel mechanism of mutation, expansion of a normally occurring polymorphic trinucleotide (CCG) repeat sequence(24). Small increases in the number of repeats were unstable premutations without significant phenotypic effect, but when these were transmitted by women they could increase dramatically in copy number, These were the full mutations that resulted in fragile X syndrome. This region of DNA was unstable somatically and when transmitted. Subsequently it has been shown by others that carriers of the premutation can have a variety of clinical conditions.

With the cloning of the fragile X we had an explanation of the Sherman paradox and a mechanism that could explain anticipation. We published a hypothesis in *The Lancet* (22) suggesting that a molecular mechanism similar to that found for fragile X could also be responsible for myotonic dystrophy and explain the anticipation that had been observed (but discredited) in that disease. Shortly after this hypothesis was published the myotonic dystrophy mutation was found to be expansion of an AGC trinucleotide repeat. Trinucleotide and other repeat expansions have now been demonstrated as the mutational mechanisms in a number of neurological disorders.

Diagnosis of fragile X syndrome was now possible by direct assay of the mutation. Family members prediagnosed for fragile X carrier status by linkage provided validation of the direct assay. On that basis, we were the first to use direct detection of the mutation for prenatal diagnosis and in most centres diagnosis of fragile X syndrome has moved from the cytogenetics to the molecular genetics laboratory.

Rob Richards decided to haplotype the fragile X chromosomes, and to my surprise, discovered the now well recognised founder effects shown by X chromosomes with long stretches of perfect CCG repeat(30). These form a reservoir in the community for the generation of new premutations.

From 1992 onwards Rob Richards and I regularly reviewed this area(36). In an earlier review(31) Rob coined the term 'dynamic mutation' to describe the mutational mechanism and this has now gained widespread acceptance.

By 1992 the Adelaide Children's Hospital had become the Women's and Children's Hospital, Adelaide, and the Cytogenetics Unit within the Department of Histopathology had become the Department of Cytogenetics and Molecular Genetics.

Other Fragile Sites

Since 1991 the positional cloning has continued under the coordination of Rob Richards. The early decision to go with chromosome 16 has paid off. Development of detailed physical and genetic maps coordinated by David Callen and John Mulley provided a base from which genes and fragile sites on this chromosome could begin to be positionally cloned. Julie Nancarrow cloned FRA16A(32) as part of her PhD project. FRA16B proved to be a more difficult project but the concerted efforts of a number of people paid off and we found a new type of sequence that could undergo dynamic mutation, an A-T rich minisatellite. FRA10B is a different type of fragile site and positional cloning proceeded from some early mapping begun by John Mulley and Liz Baker(37). Two visiting post-docs, Duncan Hewett from the UK and Oliva Handt from Germany made major contributions to the isolation of FRA10B. By collaboration with Alan Tunnacliffe and Chris Jones in Cambridge we showed that breakage at or close to FRA11B can result in Jacobsen syndrome(33).

Some fragile X families had been identified without a FRAXA mutation. I had looked at the fragile site in these families and suspected that it was distal to FRAXA. Liz Baker separated what we called FRAXE from FRAXA by FISH with probes in the area and confirmed my suspicions. Subsequently, what we had called FRAXE was further subdivided by others into FRAXE and FRAXF.

Jozef Gecz,then a senior post-doc with John Mulley was the first to isolate the gene (*FMR2*) associated with FRAXE by positional cloning(34). He demonstrated that *FMR2* is inactivated by FRAXE and this results in Fragile XE mental retardation. The first gene for a non-dysmorphic form of X-linked MR had been cloned.

Where to now?

There are still many unanswered questions about fragile sites. Their possible role in oncogenesis has again become something to consider. A common fragile site (FRA3B) is within a gene that produces abnormal transcripts in a range of solid tumours. FRA11B is within an oncogene. There are individuals in whom rare fragile sites occur on more than one chromosome and individuals with more than one fragile site on a single chromosome. These occur more often than would be expected by chance. Are there trans-acting factors (?PI) that can generate instability at multiple loci that have the potential to give rise to multiple fragile sites?

How do the DNA sequences that have been characterised at fragile sites actually give rise to the cytogenetic appearance of the fragile site? Why are fragile sites usually only seen in a

proportion of cells? Not all types of fragile site have been cloned. In particular little is known about common fragile sites. Are they areas of late replication because they contain genes being transcribed late in S phase of the cell cycle and are thus under-replicated regions of DNA that cannot package for mitosis?

Perspective

I have gained great satisfaction from the study of fragile sites. My initial work in the late 1970s provided the means for many groups to become interested particularly in the fragile X syndrome but also in the general phenomenon of fragile sites. To have been in a position to see this subject evolve, and to have participated in its evolution, from a cytogenetic curiosity to a significant area of genetics has been gratifying. I also greatly enjoyed building a research group which had a relatively large number of individuals with a wide range of talents that were all focussed, at least for part of this time, on a single project with many facets. The only down side to this for me has been that inevitably, over time, my direct input into any particular series of experiments diminished. This progression has been reflected in the authorship of papers from the group on fragile sites where I have gone from sole to first to last to second last, and into the middle.

Acknowledgements

The studies on fragile sites that are recorded here have been contributed to by all those mentioned by name, and less directly by all who have been members of the Department and its predecessors. None of the work would have been possible without the ongoing support of the current and previous administrations of the ACH and now the WCH, and their consistent view that infrastructure support for research is a vital component of a teaching hospital. I drafted this paper around the time of retirement as Head of the Department of Cytogenetics and Molecular Genetics in 2002.

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