The fragile X syndrome in a large family.

III Investigations on linkage of flanking DNA markers with the fragile site Xq27

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SUMMARY In a large family with the fragile X syndrome, we performed linkage investigations with six probes, detecting RFLPs at both sides of the fragile site Xq27. The nearest flanking markers were cX55·7 (*DXS105*) on the centromeric side ($\theta=0\cdot04$, lod 5·0) and St14 (*DXS52*) on the telomeric side ($\theta=0\cdot08$, lod 4·0).

Non-penetrance could be shown by the presence of the grandpaternal X chromosome in three mentally retarded fra(X) positive males. A second non-penetrant male in this family had inherited an abnormal grandmaternal X chromosome. His carrier mother had two retarded fra(X) positive brothers. Intermediate between the non-penetrant and fully penetrant males was a non-retarded male, who expressed the fragile site in 6% of his cells. His X chromosome showed the same polymorphisms as were found in his seven severely retarded brothers. In five fra(X) negative females the presence of an abnormal X chromosome could be demonstrated.

Despite the existence of non-penetrance in this pedigree, there was no close linkage between a factor IX polymorphism and the fragile site ($\theta=0\cdot16$, lod 1·9). However, in six descendants of a non-penetrant male, the change to penetrance appeared to be accompanied by a low recombination frequency for flanking markers.

Since the detection of the fragile site Xq27, the fragile X syndrome has increasingly confused genetic counsellors and cytogeneticists. According to Sherman *et al*,¹ no more than 56% of female carriers show cytogenetic or clinical expression and approximately 20% of affected males are missing from the expected segregation rate. These non-penetrant males, however, are able to transmit the abnormality to their daughters. Clearly, diagnostic techniques other than the conventional methods are needed to improve the detection of the abnormality.

The application of cloned genomic DNA probes, detecting restriction fragment length polymorphisms (RFLPs), has already proven to be of great diagnostic value in a number of genetic diseases in which the primary biochemical defect is still unknown. Particularly in X linked recessive diseases, like haemophilia and Duchenne muscular dystrophy, it has facilitated carrier detection and has even

become a tool for prenatal diagnosis.^{2,3} Moreover, application of DNA markers serves not only clinical purposes. The ordering of loci in the region concerned may be established from linkage data by three point segregation analysis³ and in the long term more insight into the basic defect may be obtained.

Drayna *et al*,⁴ studying the inheritance of a number of polymorphic X chromosome DNA markers, constructed the first genetic map of the X chromosome. Close linkage between the fragile site Xq27 and a factor IX probe was found by Camerino *et al*,⁵ no recombinants being reported in 15 informative meioses. However, Choo *et al*⁶ found five recombinants out of 29 meioses. Davies *et al*⁷ did not find close linkage between the DNA sequences 52A, factor IX, and the fragile site in six families. Brown *et al*^{8,9} reported linkage with 52A, factor IX, and St14 in some pedigrees but not in others, a low recombination frequency apparently depending on the presence of non-penetrant males in the pedigree.

More investigations must be performed to improve the understanding of the molecular basis of the disease and more markers closely linked to the fragile site are needed before the method becomes of clinical use. We investigated 67 members of a large family in which the fragile site Xq27 was segregating, by the application of six DNA probes,

detecting RFLPs in the distal region of the long arm of the X chromosome. The presence of a non-penetrant male in this pedigree was likely but had not been proven cytogenetically, as not all of his daughters expressed the fragile site. The cytogenetic and psychological data of this family investigation have been published in this journal.^{10 11}

FIG 1 The family pedigree: all subjects given in table 2 took part in the study on fra(X) linked RFLPs.

Materials and methods

FAMILY STUDIES

To obtain as many informative meioses as possible, 76 family members were asked to participate. All had been investigated cytogenetically¹⁰ except for IV.6 who initially refused but later consented to giving some blood. He is a normal man who finished primary and secondary school and ran his own business in horticulture. No fragile site Xq27 was found in 100 lymphocytes from his peripheral blood. The numbers in the pedigree (fig 1) indicate the 67 subjects who agreed to take part in this study.

DNA STUDIES

DNA was obtained from 30 ml of whole blood, anticoagulated with lithium-heparin. Red cells were lysed, the leucocytes pelleted by centrifuging, resuspended, and incubated with proteinase K and 1% SDS. The DNA was phenol extracted and digested with the restriction enzymes *TaqI*, *BglII*, and *MspI*. It was then separated by agarose gel electrophoresis and transferred to membrane filters by Southern blotting, as has been described by Hofker *et al.*¹²

PROBES

Six probes (table 1) were used to detect RFLPs in this family. Following nick translation and ³²P labelling the probes were hybridised to the DNA on the filters and autoradiograms were made, as described by Bakker *et al.*³

The probes 52A (*DXS51*) and factor IX have been localised on the centromeric side of the fragile site.^{13 14} The probe cX55-7 (*DXS105*), developed by Hofker *et al.*¹⁵ is also located on the centromeric side, closely linked to the fragile site.^{15 16} It detects a *TaqI* polymorphism defined by two alleles with fragment sizes of 4.2 and 3.2 kb.

The probes St14 (*DXS52*) and DX13 (*DXS15*) detect RFLPs on the telomeric side.^{4 17} St14 recognises at least six *TaqI* alleles, numbered T1 to T6. They can be distinguished by a 5.0, 4.8, 4.5, 4.3, and 4.1 kb band respectively, flanked by invariable bands of 5.4 and 3.8 kb (T1 to T5), or a 3.5 kb band

outside the constant band of 3.8 kb (T6). The probe cpX67 (*DXS134*) has also been localised on the telomeric site of the fragile Xq27.¹⁵ It detects an *MspI* polymorphism defined by two alleles of 3.7 and 3.4 kb.

LINKAGE ANALYSIS

Lod scores were computed using the LINKAGE computer programme.¹⁸ Input parameters used in the linkage analysis were a gene frequency of 0.0006, a mutation rate of 0.00036, and a penetrance in males of 0.80 and in females of 0.56 as calculated by Sherman *et al.*¹ Males were considered to be fra(X) negative if no more than 1/100 cells showed the abnormality; for females the cut off point was 2/100 cells.^{10 11 19}

Results

All daughters of III.12 inherited the polymorphisms a-K-T₄-c-j from their father and either h-A-k-T₅-B-J or H-a-K-T₄-B-J or a cross over chromosome from their mother (table 2). In generation V, three mentally retarded fra(X) positive males (V.57, 63, and 67) appear to have inherited the grandpaternal X chromosome from their mothers. Therefore, III.12 must have been a transmitting male, all his daughters necessarily being carriers.

All identified RFLP alleles for the probes used are summarised in table 2. Fig 2 shows the pedigree together with the percentages of fra(X) positive cells. Recombination events are indicated with x or x-x if the region of recombination was less distinct. On account of the inherited markers, five cytogenetically negative females (IV.40, 42, 48, 50, and V.71) and one male of normal intelligence with only one in 100 fra(X) positive cells (V.76) must have inherited the abnormality.

Lod scores were calculated at intervals of a genetic distance of 5 cM ($\theta=0.05$). The linkage data on this family (excluding IV.52 and her daughters, who strictly speaking belong to a second fra(X) family) between the six DNA markers and the fra(X) are presented in table 3. Mutual linkage between the markers has also been analysed. The maximum lod scores and 95% confidence limits of various thetas are given in table 4.

No positive lod scores between the fra(X) and 52A (*DXS51*) were found at any distance. A peak lod score of 1.9 was found for linkage between fra(X) and the factor IX probe, with a recombination frequency of 16%. A peak lod score of 5.0 was calculated for linkage with cX55-7 (*DXS105*) at $\theta=0.04$. This marker is not only closely linked to the fragile site but also to factor IX ($\theta=0.08$, lod 3.9). It appears to be located between these two loci on the proximal side of the fragile site. The probability of

TABLE 1 Probes used in the study.

Probe	Restriction enzyme	Reference	Code
52A (<i>DXS51</i>)	<i>TaqI</i>	4	H/h
cVIII (F9)	<i>TaqI</i>	13	A/a
cX55-7 (<i>DXS105</i>)	<i>TaqI</i>	15	K/k
St14 (<i>DXS52</i>)	<i>TaqI</i>	17	T1-6
DX13 (<i>DXS15</i>)	<i>BglII</i>	4	B/b
cpX67 (<i>DXS134</i>)	<i>MspI</i>	15	J/j

FIG 2 The polymorphisms and the percentages of fra(X) positive cells are shown for each subject. H/h=52A (DXS51). A/a=F9 (cVIII). K/k=cX55.7 (DXS105). T1-6=S14 (DXS52). B/b=DX13 (DXS15). J/j=cpX67 (DXS134).

the order FIX-cX55-7-fraX-St14 against cX55-7-FIX-fraX-St14 is more than 100 to 1. The results of the LINKMAP analysis (table 5) confirm the gene order deduced from the pedigree in which the physical order of the markers was established by analysis of multipoint crosses.

TABLE 2 Identified RFLP alleles.

Pedigree No	Normal or MR	% fra(X)	Probes					
			52A	F9	cX557	St14	DX13	cpX67
IV.2	MR	28	—	a	K	T ₂	b	j
IV.4	N	8	—	Aa	KK	T ₁ T ₂	BB	JJ
IV.6	N	0	—	a	k	T ₅	B	J
IV.7	MR	19	—	a	K	T ₂	b	j
IV.8	MR	19	—	a	K	T ₂	b	j
IV.9	MR	30	—	a	K	T ₂	b	j
IV.10	N	6	—	a	K	T ₂	b	j
IV.11	MR	40	—	a	K	T ₂	b	j
IV.12	MR	28	—	a	K	T ₂	b	j
IV.38	N	0	—	A	k	T ₅	B	J
IV.40	N	0	—	Aa	Kk	T ₄ T ₅	Bb	Jj
IV.41	N	6	—	Aa	Kk	T ₄ T ₄	Bb	Jj
IV.42	N	0	Hh	Aa	KK	T ₄ T ₄	Bb	Jj
IV.46*	N	0	—	A	k	T ₄	B	J
IV.46	N	9	—	Aa	KK	T ₄ T ₄	Bb	Jj
IV.47	N	0	H	a	K	T ₅	B	J
IV.48	N	2-4	Hh	Aa	Kk	T ₄ T ₅	Bb	Jj
IV.49	N	9	Hh	Aa	Kk	T ₄ T ₅	Bb	Jj
IV.50	N	0	HH	aa	KK	T ₄ T ₄	Bb	Jj
IV.51	N	0	h	A	k	T ₅	B	J
IV.52	N	5	—	Aa	Kk	T ₅ T ₅	Bb	Jj
IV.55	MR	9	H	a	K	T ₄	B	J
IV.56	N	0	H	A	k	T ₅	b	j
IV.57	N	0	H	A	k	T ₄	B	J
IV.58	N	20	Hh	Aa	KK	T ₄ T ₅	Bb	Jj
IV.59	N	18	Hh	Aa	KK	T ₅ T ₅	bb	jj
IV.60	N	0	Hh	AA	Kk	T ₅ T ₅	bb	Jj
IV.61	N	0	Hh	AA	Kk	T ₅ T ₅	bb	Jj
IV.62	N	0	h	A	k	T ₅	b	j
IV.63	MR	9	H	a	K	T ₄	B	J
V.53	N	0	—	A	K	T ₄	b	j
V.54	N	4	hh	AA	Kk	T ₄ T ₄	BB	JJ
V.55	N	4	—	—	Kk	T ₄ T ₄	Bb	Jj
V.56	MR	20	—	AA	Kk	T ₄ T ₄	Bb	Jj
V.57	MR	13	H	a	K	T ₄	b	j
V.59	MR	21	—	Aa	Kk	T ₄ T ₄	Bb	Jj
V.60	N	0	HH	AA	kk	T ₅ T ₅	Bb	Jj
V.62	MR	24	Hh	AA	kk	T ₂ T ₄	Bb	Jj
V.63	MR	18	—	a	K	T ₄	b	j
V.64	N	0	H	A	k	T ₅	B	J
V.67	MR	50	H	a	K	T ₄	b	j
V.68	N	0	H	a	—	T ₄	B	—
V.71	N	0	Hh	Aa	Kk	T ₅ T ₅	Bb	Jj
V.72	N	3	hh	Aa	Kk	T ₅ T ₅	Bb	Jj
V.73	N	0	hh	aa	kk	T ₅ T ₅	BB	JJ
V.74	N	8	HH	Aa	KK	T ₄ T ₅	BB	Jj
V.77	N	26	HH	Aa	KK	T ₄ T ₅	BB	Jj
V.75	N	0	Hh	AA	KK	T ₅ T ₅	Bb	jj
V.76	N	2	H	a	K	T ₄	B	J
V.78	N	0	Hh	Aa	KK	T ₅ T ₅	Bb	Jj
V.79	MR	14	h	A	K	T ₅	b	j
V.80	N	0	Hh	Aa	kk	T ₂ T ₅	Bb	JJ
V.81	N	0	hh	Aa	kk	T ₂ T ₅	Bb	JJ
V.82	N	0	hh	Aa	kk	T ₂ T ₅	Bb	JJ
VI.110	N	0	—	A	k	T ₄	B	J
VI.121	N	0	—	A	k	T ₄	b	j
VI.122	N	0	h	A	k	T ₄	b	j
VI.161	N	0	H	A	K	T ₅	b	j
VI.162	N	0	hh	AA	Kk	T ₅ T ₅	Bb	Jj
VI.151	N	0	—	AA	Kk	T ₂ T ₅	Bb	Jj
VI.152	N	0	H	—	K	T ₅	B	j
VI.153	N	0	H	A	K	T ₅	B	j
VI.171	N	0	HH	Aa	Kk	T ₅ T ₆	BB	Jj
VI.172	N	0	HH	Aa	Kk	T ₅ T ₆	BB	Jj
VI.181	N	0	—	aa	—	T ₁ T ₅	Bb	—
VI.191	N	0	hh	Aa	kk	T ₂ T ₅	Bb	Jj

*Husband

TABLE 3 *Lod scores: fragile X with other markers.*

	Recombination distance								
	0-01	0-06	0-11	0-16	0-21	0-26	0-31	0-36	0-41
Fra(X)	-3.798	-2.258	-1.484	-1.007	-0.687	-0.467	-0.315	-0.206	-0.123
52A									
Fra(X)	-0.419	1.305	1.807	1.934	1.877	1.707	1.454	1.137	0.764
F9									
Fra(X)	4.762	4.963	4.655	4.224	3.722	3.166	2.563	1.917	1.231
cX55-7									
Fra(X)	3.099	4.015	3.984	3.724	3.339	2.865	2.316	1.702	1.043
St14									
Fra(X)	-2.014	0.663	1.396	1.666	1.695	1.570	1.333	1.021	0.662
DX13									
Fra(X)	-3.525	-0.503	0.517	1.004	1.210	1.229	1.088	0.880	0.578
cpX67									

The nearest markers on the telomeric side are St14 (*DXS52*) at $\theta=0.08$ (lod score 4.0), followed by DX13 (*DXS15*) at $\theta=0.19$ (lod 1.7), and finally by pX67, which appeared to be closely linked with DX13 at $\theta=0.08$, lod 5.5.

TABLE 4 *Two point recombination fractions, maximum lod scores, and 95% confidence limits.*

		θ	lod	95% confidence limits θ
Fra(X)	FIX	0.165	1.935	0.042-0.388
	cX55-7	0.039	5.011	0.001-0.182
	St14	0.079	4.004	0.001-0.242
	DX13	0.192	1.706	0.062-0.404
	cpX67	0.241	1.241	0.092-0.461
cX55-7	FIX	0.079	3.968	0.011-0.243
	St14	0.162	2.599	0.055-0.333
	DX13	0.222	0.879	Undetermined
	cpX67	0.260	0.895	Undetermined
	52A	0.225	1.184	0.087-0.450
F9	St14	0.246	1.272	0.102-0.464
	DX13	0.233	1.001	0.083-0.500
	cpX67	0.298	0.848	Undetermined
	St14	0.150	2.332	0.039-0.349
	DX13	0.080	5.598	0.017-0.212
St14	DX13	0.053	4.504	0.001-0.208

TABLE 5 *Lod scores for three point linkage analysis.*

Order is cX55-7-factor IX-fra(X).

Recombination distance is fixed at $\theta=0.08$ for cX55-7 to factor IX and is allowed to vary between factor IX and fra(X).

	Recombination fraction (θ)									
	0-06	0-07	0-08	0-09	0-10	0-11	0-12	0-13	0-14	0-15
Lod score	18.06	18.59	18.96	19.22	19.38	19.46	19.47	19.42	19.32	19.18

Order is factor IX-cX55-7-fra(X).

Recombination distance is fixed at $\theta=0.08$ for cX55-7 to factor IX and is allowed to vary between cX55-7 and fra(X).

	Recombination fraction (θ)							
	0-03	0-04	0-05	0-06	0-07	0-08	0-09	0-10
Lod score	22.48	22.85	23.07	23.17	23.19	23.14	23.04	22.89

Discussion

The development of the probe cX55-7 detecting a DNA polymorphism between factor IX and the Xq27 fragile site⁴ not only serves a diagnostic purpose, but may also increase our understanding of the initiation and progression of the fragile site itself. Further investigations are needed to confirm that the genetic distance between cX55-7 and fra(X) (q27) is only about 4 cM.

On the telomeric side the nearest marker was St14 with a peak lod score of 4.0 at a distance of 8 cM from the fragile site. This is a closer linkage than has been reported by Forster-Gibson *et al.*,²⁰ who found $\theta=20$ (lod 1.84) in 10 families and by Brown *et al.*,⁹ who established a $\theta=15$ (lod 4.51) in 16 families of their own and 16 families reviewed from published reports. However, Oberle *et al.*,²¹ investigating another 16 families, found the same distance between St14 and the fragile site as we did. Because of this variability in different families, the finding of closely linked flanking markers becomes even more important.

Polymorphism for both flanking markers was found in the carrier females IV.40, 48, and 49, who showed the fra(X) in 0, 2, and 9% of cells respectively. The improvement in diagnostic capacity is obvious. However, the frequency of the minor allele of cX55.7 is only 20%. The heterozygosity at the locus must be increased by subcloning of flanking sequences to extend the polymorphic information content²² and investigations to achieve this are under way. Application of the method to chorionic villi samples could make prenatal diagnosis more reliable and acceptable.

The other newly developed probe, cpX67, appears to be located on the telomeric side of the fragile site at $\theta=24$ (lod 1.2). Its application in families with the fragile X syndrome is not expected to be of great diagnostic value. This marker, however, may be helpful in establishing the order of loci in the region concerned. A precise location of the haemophilia A gene is not yet known. Meiotic recombination has been reported between factor VIIIc and DX13^{23, 24} and also between factor VIIIc and St14,^{25, 26} one family showing a cross over between the haemophilia locus and both DX13 and St14. This would imply that the factor VIIIc gene is not located between, but outside, these two markers, in agreement with a recent report by Connor *et al.*²⁷ Application of cpX67 may help to establish whether the gene is on the centromeric or the telomeric side.

In our pedigree, linkage analysis provided more information about the origin, presence, and inheritance of the abnormality than could be achieved by cytogenetic methods alone. The presence of five carrier females without cytogenetic expression (IV.40, 42, 48, 50, and V.71) could be shown. Though two of them were obligate carriers in whom the diagnosis was easy, the recognition of the others had important implications. Only by linkage analysis with RFLPs was it possible to prove that III.12 had been non-penetrant, that is, a normal transmitting male. Consequently, all his daughters were carriers. In the offspring of these daughters there is a remarkably high proportion of mentally retarded males and females: seven out of nine females (77%) inherited the fra(X), five of them (71%) being retarded; five out of eight males expressed the fra(X) and were also retarded. The reported numbers are small but strongly disagree with Steinbach's model for the prediction of mental deficiency in the fragile X syndrome.²⁸ The model assumes that the retardation is determined not only by an X linked gene at or near the fragile site, but also by at least one autosomal modifier gene. It predicts for the offspring of daughters of a normal transmitting male a risk of about 8% of a mentally

retarded daughter and of 34% of a mentally retarded son, which substantially differs from the findings in the daughters of III.12.

Another male in this pedigree (V.76), who was not mentally retarded, expressed the fragile site in only 1/100 cells. He had been considered fra(X) negative, but appeared to have inherited the abnormal X chromosome. Two of his sisters, having inherited the same X chromosome from their mother, expressed the fragile site in 9% and 20% of their cells, respectively. The mother herself was also an expressing carrier and had two mentally retarded fra(X) positive brothers. This male represents the second case of non-penetrance in this pedigree. His case demonstrates that not only progression of the abnormality from non-penetrance to penetrance may occur, but also the reverse. It means that there probably is heterogeneity in mothers of non-penetrant males.

Intermediate between the two non-penetrant males and the fully penetrant male patients is the non-retarded man (IV.10), who expressed the fra(X) in 6/100 of his cells. Such quantitative differences in penetrance could fit with the hypothesis of Nussbaum *et al.*²⁹ which postulates that expression rates depend on the extent of the amplification of pyrimidine rich DNA sequences. It implies that repair mechanisms may cause the disappearance of the abnormality, resulting in non-penetrance, as in V.76. Partial penetrance, as in IV.10, could then be explained by partial repair. This would fit in with the theory of Pembrey *et al.*³⁰ that a stepwise progression of a genetic change, probably resulting from duplication or deletion, generates the fra(X) mutation. The mechanism for such a process may be found in misalignment and repair might simply mean a deletion of the amplified sequences.

Yet another non-retarded male in this family (V.68), whose mother was only informative for DX13 and cpX67, showed the fragile site in 1/100 cells. However, he seems to have inherited the normal X chromosome, which throws doubt upon the diagnostic meaning of the cytogenetic result. From these data we conclude that the choice of a low cut off point (2%) to evaluate the cytogenetic results is of little clinical value; moreover, this value takes no account of false negatives. It also appears that without closely linked markers it is almost impossible to distinguish between penetrant and non-penetrant fra(X) families. Non-penetrance will remain unnoticed unless the family history indicates otherwise. In the case of V.76 no indication for non-penetrance, other than the RFLP data, was present.

We have already mentioned the data indicating a rather loose linkage between 52A (DXS51) and the

fragile site.⁷⁻⁹ In our pedigree no positive lod scores could be demonstrated. Various authors have reported considerable differences in genetic distances between factor IX and the fragile site.^{5-8, 31} This has been attributed to genetic heterogeneity, the two classes of pedigrees being distinguished by the presence of either penetrant or non-penetrant males.^{8, 9} However, in our pedigree, recombination between factor IX and the fra(X) or St14 in descendants of the non-penetrant male III.12 occurred. No less than 15 crossovers could be shown in 25 of his offspring. This constitutes an exception to the suggestion^{8, 9} that a closer linkage exists between fra(X) and flanking markers in non-penetrant pedigrees.

Differing estimations of genetic distances are not exceptional. A comparable divergence, for instance, can also be found for various sets of linkage data of DXS84 (754) with Duchenne muscular dystrophy on the short arm of the X chromosome.³² In most cases it is difficult to answer the question whether such differences indicate genetic heterogeneity or just sampling variation. In the case of the fra(X) syndrome, insufficient data on the presence of non-penetrance in some of the reported pedigrees could easily bias the results.

Pembrey *et al.*³⁰ have drawn attention to the fact that transmission through a female carrier seems to be necessary to generate the fully penetrant form of the fra(X) mutation. In a recent paper, Winter and Pembrey³³ suggested that support for their theory of (double) recombination or gene conversion within the fra(X) region being necessary for the difference between penetrant and non-penetrant expression could be found by examining recombination frequencies in adjacent sequences. Classic meiotic theory would predict that occurrence of recombination within one segment would probably lead to suppression of recombination in neighbouring sequences by interfering. In the fra(X) concept proposed by Winter and Pembrey³³ this would result in lower recombination frequencies in adjacent sequences in the same meiosis giving rise to penetrant males.

We studied the data on 12 children, born to four daughters of III.12: six males and six females. Three males expressed the fragile site and were mentally retarded: non-penetrance had changed into full cytogenetic and clinical penetrance (V.57, 63, 67). Three of the females were also mentally retarded (V.56, 59, 62). They expressed the fragile site in considerably larger percentages than two non-retarded carriers (V.54, 55). One woman was normal (V.60). In the six retarded subjects, one crossover had occurred in the region of the fragile site itself (V.62) and one crossover outside the region between 52A and factor IX (V.56), whereas

in the six subjects without a change in penetrance (the normal woman included), seven meiotic recombinations could be demonstrated. Although these numbers are too small to draw conclusions, the ratio one or two to seven seems compatible with the hypothesis mentioned above.

For scientific and clinical reasons the promising investigations with recombination DNA methods must be continued actively, to solve the numerous questions about the fragile X syndrome.

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