A promoter mutation in the *XIST* gene in two unrelated families with skewed X-chromosome inactivation

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X-chromosome inactivation is the process by which a cell recognizes the presence of two copies of an X chromosome early in the development of XX embryos and chooses one to be active and one to be inactive[1]. Although it is commonly believed that the initiation of X inactivation is random, with an equal probability (50:50) that either X chromosome will be the inactive X in a given cell, significant variation in the proportion of cells with either X inactive is observed both in mice heterozygous for alleles at the *Xce* locus[2] and among normal human females in the population[3–5]. Families in which multiple females demonstrate extremely skewed inactivation patterns that are otherwise quite rare in the general population are thought to reflect possible genetic influences on the X-inactivation process[5–7]. Here we report a rare cytosine to guanine mutation in the *XIST* minimal promoter that underlies both epigenetic and functional differences between the two X chromosomes in nine females from two unrelated families. All females demonstrate preferential inactivation of the X chromosome carrying the mutation, suggesting that there is an association between alterations in the regulation of XIST expression and X-chromosome inactivation.

As a critical part of the X-inactivation process, the X-inactivation center (XIC) has been implicated in recognizing the number of X chromosomes and initiating a signal that then propagates in *cis*, silencing most of the genes on the associated X chromosome. The *XIST* gene[8–10], which maps to the XIC region on both human and mouse X chromosomes, does not encode a protein, is expressed exclusively from inactive X chromosomes and is an excellent candidate for a gene involved in the initiation or establishment and promulgation of the inactivation signal[8,10,11]. Although recent reports from mouse studies suggest that expression of the *Xist* gene is both necessary and sufficient for X inactivation[12–15], the possible effects of mutations in the human *XIST* gene are unknown.

Previous studies of unrelated females have shown that X-inactivation patterns follow a gaussian distribution, with most females demonstrating inactivation patterns (that is, the proportion of cells with either X inactive) ranging from 50:50 to 80:20; only about 1% of females exhibit patterns as skewed as 95:5 or greater[3,4,6,16]. Although initiation of X inactivation is thought to be random, the broad distribution of X-inactivation patterns among unrelated females is thought to be the result of stochastic fluctuation involving a small number of progenitor cells[3,17]. Alternatively, it is possible that at least some females with skewed X-inactivation patterns represent a genetically distinct class predisposed to skewed X inactivation.

In principle, genetic influences on X-inactivation patterns could mean that an X is chosen to be active or could apply to the relative rates of growth of the two cell populations after X inactivation has been established[17]. The only known example of a primary genetic effect on X inactivation is observed in mice heterozygous for the X-controlling element (*Xce*) locus, in which there is preferential inactivation of the chromosome carrying the weaker *Xce* allele[2]. Although there appears to be an inverse correlation between the level of *Xist* expression and the strength of the closely linked *Xce* allele, this correlation is imperfect, and the exact molecular nature of the *Xce* effect, as well as its relationship to the *Xist* gene, is likely to be complex[10,18,19]. In humans, families have been identified in which multiple females demonstrate significantly skewed X-inactivation patterns that are not obviously explained by stochastic fluctuation or secondary cell selection, suggesting that genetic factors may influence the initial choice of which X is to remain active and which X is to become inactivated[6,7].

To investigate this possibility, we have undertaken a study to identify families in which multiple females demonstrate inactivation patterns of at least 95:5, and to subsequently determine the basis for such familial skewing. Individuals I-1 and II-1 from family I (Fig. 1*a*) were previously described[20] as exhibiting extremely skewed X inactivation in multiple tissues, with X-inactivation patterns greater than 95:5. Although several females in this family are obligate or proven carriers for a 1.9-Mb deletion of the steroid sulphatase (*STS*) gene characteristic of most cases of X-linked ichthyosis[21], the deletion appears to segregate independently of skewed X inactivation in this family (for example, individual II-3, Fig. 1), and similar or even larger deletions are associated with random X-inactivation patterns in other families[22]. Thus, we sought other genetic explanations for the observed skewed X inactivation in this family.

Analysis of expression of the androgen receptor (*AR*) gene in fibroblast cell cultures from individuals I-1 and II-1 confirmed preferential X inactivation in these cells and identified the shared allele residing on the preferentially inactive X chromosome in both females (Fig. 1*b*; allele 'B'). Controls mixing various amounts of input RNA demonstrated that this expression assay was sensitive enough to detect allelic ratios as extreme as 97:3 (data not shown). To quantitate the proportion of cells with one or the other X chromosome inactive in each available family member, we analysed fibroblast or lymphocyte DNA at the *AR* or fragile X mental retardation (*FMR1*) loci, using methods based on the differential methylation of sites within *AR* or *FMR1* on the active or inactive X chromosome[16,23]. Using this approach, we determined that individuals I-1 and II-1 displayed inactivation patterns greater than 95:5 (validating the results of the *AR* expression assays, as well as other X-inactivation assays[20]), whereas all other family members demonstrated X-inactivation patterns more typical of those seen frequently in the general population (Fig. 1*c*; refs 4,6,16,23).

As haplotype analysis demonstrated that individuals I-1 and II-1 share the same XIC on their respective preferentially inactive

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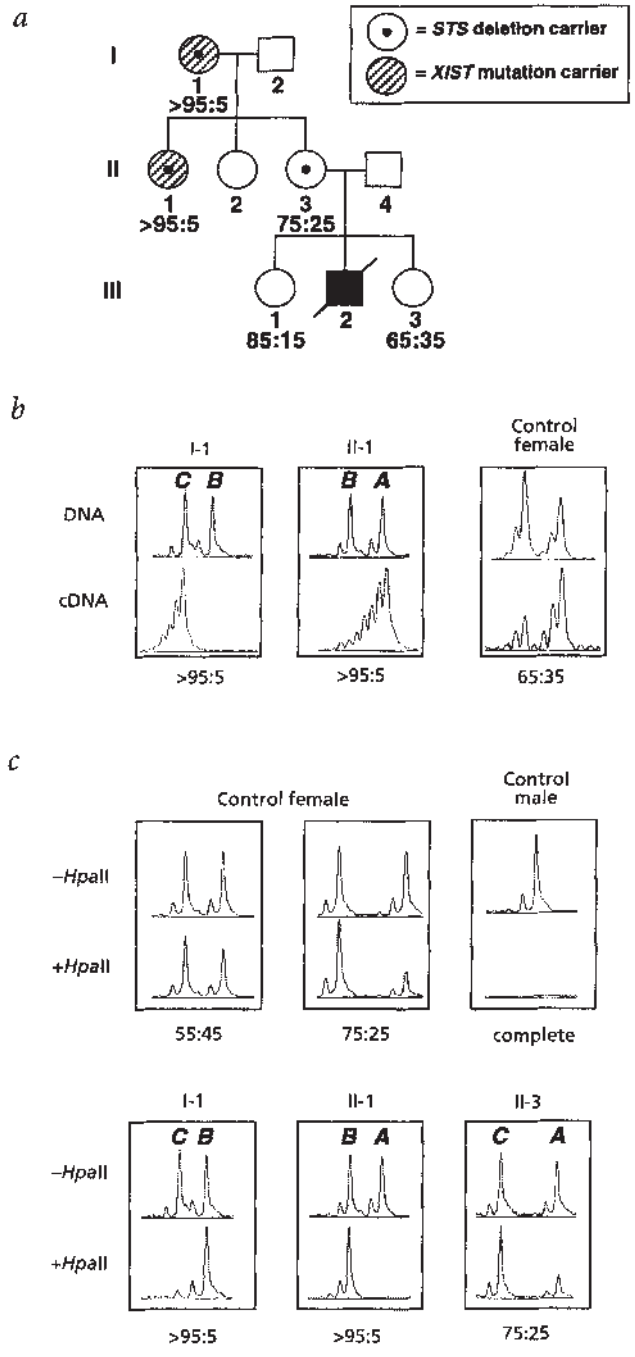

Fig. 1 Familial skewed X-chromosome inactivation. **a**, Pedigree of family I. Ratios represent X-chromosome inactivation patterns, determined by testing at the *AR* and *FMR1* loci. Individuals I-1, II-1 and II-3 are carriers of a 1.9-Mb deletion of the steroid sulphatase (*STS*) gene[20,21]. Individual III-2 was affected with ichthyosis. Hatched symbols indicate carriers of the *XIST*-43 mutation. **b**, Fibroblast RNA from individuals I-1 and II-1 was analysed to determine the randomness of X inactivation. The relative expression level of alleles at *AR* were compared by quantitative RT-PCR. Alleles arbitrarily designated 'C' and 'A' were preferentially expressed in individuals I-1 and II-1, respectively, at a level of >95:5; the shared 'B' allele is thus on the preferentially inactive X in both females. The third panel shows the tracing from an unrelated female with an inactivation pattern of 65:35. **c**, With a differential methylation assay, X-inactivation patterns were calculated for individuals informative for this *AR* polymorphism by comparing the values of the intensities of the alleles amplified from *Hpa*II-digested DNA (lower tracings, each panel), normalized to the values obtained from non-digested DNA (upper tracings, each panel). X-inactivation patterns from two control females and one control male are shown in the top row. Male DNA served as a control for complete *Hpa*II digestion. Tracings from three members of family I are shown in the bottom row. Individual III-3 was not informative at the *AR* locus, and her inactivation pattern was determined by analysis at the *FMR1* locus[23]. DNA was not available for II-2.

X chromosomes (data not shown), we considered *XIST* to be an attractive candidate gene underlying the skewed inactivation, and we sought alterations in *XIST* by sequencing its most highly conserved regions. At the 5′ end of the gene are two such regions, the minimal promoter of approximately 125-bp and a series of transcribed 43–59-bp direct repeats (Fig. 2*a*; refs 24,25). We sequenced 1.7 kb of this region in individuals I-1 and II-1 and a series of controls and identified a single C to G mutation at position –43 in the minimal promoter on the preferentially inactive X in individuals I-1 and II-1, but not in other family members (Fig. 2*b*). No other mutations or polymorphisms were identified in the 1.7-kb region examined. Analysis of mouse–human somatic-cell hybrids derived from individual I-1 (and from II-1; data not shown) demonstrated that the mutation resides on the preferentially inactive X in this family (Fig. 2*c*).

The –43 mutation creates a novel *Hha*I restriction site that was used to test for the presence of the mutation in a large series of unrelated individuals, representing 1,166 independent X chromosomes (Fig. 2*c*). The mutation was found on only one additional chromosome in this data-set, ruling out that the mutation represents a common polymorphism. The heterozygous female identified by this screen is from a large family (family II) with Snyder-Robinson syndrome[26], a form of X-linked mental retardation that maps to Xp21.3–22.12 (Fig. 3). The haplotypes of several polymorphisms around the XIC region were compared in families I and II, and no common alleles were identified, indicating that these families do not share recent ancestors (data not shown). Subsequent analysis of family II revealed that six additional females and four males also inherited the *XIST* mutation (Fig. 3). The X-inactivation patterns of the seven *XIST* mutation carriers in family II ranged from 55:45 to more than 95:5 (Figs. 3,4*b*). Four of the seven were also carriers of the Snyder-Robinson mutation (Fig. 3). By following the segregation of the *XIST* promoter mutation and differentially methylated *AR* alleles, we determined the mutation to be on the preferentially inactive X chromosome in all seven females (data not shown).

To examine further the association between the *XIST* promoter mutation and skewed X inactivation noted in families I and II, we compared the X-inactivation patterns of females with and without the *XIST* promoter mutation. The distribution of X-inactivation patterns of 115 unrelated females, none of whom carries the *XIST* mutation (Fig. 4*a*), is similar to that reported by others using the same or similar assays[4,6,16]. If the *XIST* mutation has no effect on X-chromosome inactivation, one would predict that the mutant allele would reside on the preferentially active or inactive X chromosome at approximately equal frequencies. In families I and II, however, all nine females demonstrate preferential inactivation of the mutant X chromosome ($P < 0.01$; Fig. 4*b*).

To determine whether this mutation affects activity of the *XIST* promoter, a 237-bp fragment (positions –209 to +28) from either the mutant or wild-type *XIST* promoter was cloned in front of a promoterless luciferase reporter gene, transiently transfected into both male (the HT1080 fibrosarcoma line) and female (the 293 embryonic kidney line) human tissue culture cells and assayed for luciferase activity as described[25]. In this assay, the mutant promoter construct, pGLXm, consistently produced two- to fivefold lower levels of luciferase activity (0.17 ± 0.11; $n = 18$) than the normal promoter construct, pGLXH (1.0 ± 0.38, $n = 28$), thereby establishing a functional difference between the normal and mutant promoters.

Stochastic factors are generally considered to be responsible for the broad distribution of X-inactivation patterns observed among

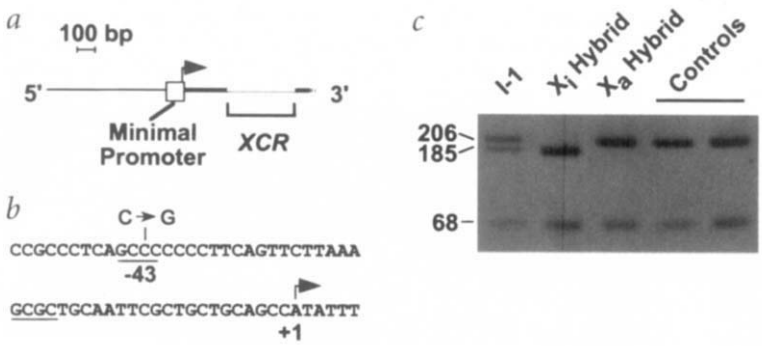

Fig. 2 Identification and functional characterization of a C→G mutation in the *XIST* minimal promoter. ***a,b***, Sequence analysis of 1.7 kb of the 5′ end of the *XIST* gene identified a C→G mutation at position –43 relative to the start of transcription (arrow) in the *XIST* minimal promoter of individuals I-1 and II-1. Both a novel *Hha*I restriction site created by the mutation and a control *Hha*I site in the minimal promoter are underlined. XCR indicates a series of conserved repeats in the *XIST* gene[25]. **c**, Amplification of a 276-bp fragment from the minimal promoter and subsequent *Hha*I digestion were performed on DNA from individual I-1, from mouse–human somatic-cell hybrids derived from individual I-1 containing either a single active or inactive X and from 1,166 unrelated X chromosomes. After digestion, the normal promoter yields 206-bp and 68-bp fragments; the mutant promoter yields 185-bp, 68-bp and 21-bp fragments. Individual I-1 is heterozygous for this *Hha*I site. Based on analysis of DNA derived from mouse–human somatic cell hybrids, the mutation was present on the preferential inactive X chromosome in individual I-1.

normal females in the general population. Three lines of evidence suggest, however, that genetic factors may also contribute to this distribution. First, post-inactivation selection against cells containing certain X-linked mutations on the active X has been well documented[5,17]. Second, mice heterozygous for *Xce* alleles demonstrate non-random X inactivation, suggesting that similar alleles may be present in humans. Although the association noted here between reduced *XIST* promoter activity and preferential X inactivation is the opposite of trends noted for mice heterozygous at the *Xce* locus[10,18,19], in the absence of further mechanistic information one would not necessarily expect a direct parallel between the murine *Xce* and human *XIST* mutations. And third, families have been described in which multiple females demonstrate skewed X-inactivation patterns not easily explained by stochastic factors or obvious secondary cell selection[6,7]. One hypothesis is that these families harbour X-linked alleles that result in preferential inactivation of the associated X chromosome, and the *XIST* promoter mutation is a candidate for such an allele.

It is statistically unlikely that all nine females from families I and II would demonstrate preferential inactivation of the chromosome carrying the *XIST* mutation simply due to chance ($P<0.01$). However, whether this association is due to the *XIST* mutation alone or to the co-inheritance of the *XIST* mutation and a mutation at an X-linked disease locus is unclear, particularly because other reports have indicated an association between skewed X inactivation and carriers of mutations at other X-linked mental-retardation loci[27–29]. This uncertainty is most evident in family II, in which four of the seven females carry both the *XIST* promoter mutation and a Snyder-Robinson syndrome mutation. The range of observed X-inactivation patterns in family II is 55:45 to more than 95:5 (Fig. 4*b*), with the three most extreme patterns occurring in females who carry both mutations. This raises the possibility that the skewed inactivation observed in family II is related, at least in the most extremely skewed cases, to the presence of both mutations. It is thus possible that the *XIST* mutation predisposes to preferential X inactivation of the associated chromosome, and that additional X-linked mutations may compound the skewed inactivation phenotype.

If the *XIST* mutation is responsible for preferential X inactivation, by what mechanism does it occur? At least conceptually, the first step in the inactivation process entails recognition of the number of X chromosomes present and (in the case of a normal female with two X's) silencing the *XIST* allele on the X that will remain active in each cell[25,30]. It is plausible that the mutation in the *XIST* promoter influences this process by distinguishing between the two *XIST* genes present in heterozygotes. The subsequent choice between the two *XIST* genes may be biased by either a DNA-based effect (that is, the promoter sequences are different) or an RNA-based effect (that is, the level of *XIST* transcripts may be different). Alternatively, the promoter mutation may result in differential growth of the two cell populations carrying the mutant *XIST* gene on either the active or inactive X; the potential growth advantage of cells with the mutation on the inactive X may be heightened in females who also carry a mutation in another gene on the same X. The basis for this post-inactivation cell selection is not immediately obvious, but it could involve alterations in either the timing or extent of X inactivation at a critical stage of cell proliferation in early development.

The identification of a mutation in the *XIST* minimal promoter in two unrelated families with skewed X inactivation suggests that the regulation of *XIST* expression contributes to the variability of X-inactivation patterns observed among females in the general population, as well as among females who are known carriers for X-linked diseases. The existence of this mutation supports a model in which events involving the *XIST* promoter influence an individual's X-inactivation pattern and provides evidence in humans for the now established role of the mouse *Xist* gene in X inactivation[12,14,15,31,32]. Additional studies, either in other families carrying identical or similar mutations or in transgenic mice with mutations introduced into the *Xist* promoter, will be necessary to confirm this association and for us to understand its basis.

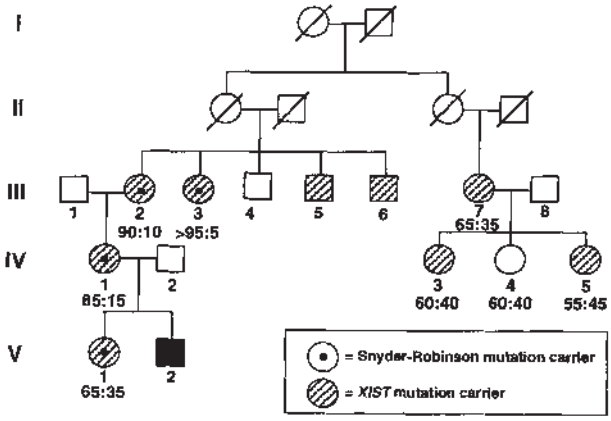

Fig. 3 *XIST* mutation in family II. The –43 mutation (hatched symbols) was detected in seven females and four males (III-5, III-6 and two sons of III-3 not shown on the pedigree). Individual V-2 is affected with Snyder-Robinson syndrome[26]. The X-inactivation pattern of each female was determined with the *AR* methylation assay (Fig. 1*c*).

Methods

***AR* X-inactivation patterns.** To determine the relative expression of alleles at the *AR*, we modified a previously described protocol[33]. Total RNA (2 µg) was reverse transcribed with the *AR*-RT primer (5′–GGCTCCAGGCTC-TGGGACGCAACCTCT–3′; 1 µM) in a 20-µl reaction containing dNTP (100 µM each), 1× First Strand Buffer (Gibco BRL; 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 200 U M-MLV RT

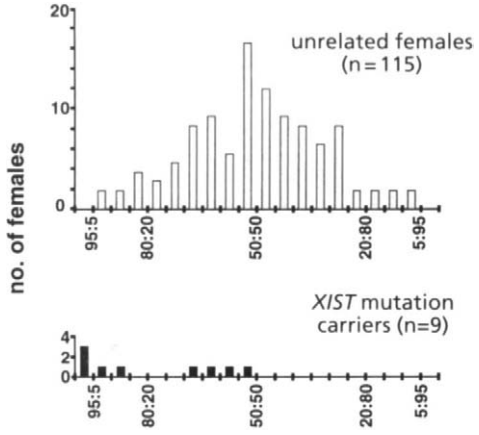

Fig. 4 Distribution of X-inactivation patterns determined with the *AR* methylation assay. ***a***, X-inactivation patterns of 115 unrelated females without the *XIST* mutation. The X-inactivation patterns are arbitrarily expressed as a ratio of the larger *AR* allele to smaller *AR* allele. ***b***, X-inactivation patterns in nine female carriers of the *XIST* mutation from families I and II. The X-inactivation patterns are expressed as the ratio of the mutant to normal X chromosome.

(GIBCO BRL), and 20 U RNase Inhibitor (GIBCO BRL). Reverse transcription was performed for 1.5 h at 42 °C, followed by a 10-min inactivation step at 95 °C. The RT reaction (1 µl) was added to a PCR reaction containing 1× PCR Buffer (GIBCO BRL; 20 mM Tris (pH 8.4), 50 mM KCl), 1.5 mM $MgCl_2$, 150 µM c^7dGTP, 50 µM dGTP, 200 µM dATP, 200 µM dTTP, 200 µM dCTP, 3% DMSO, 0.01% gelatin, 0.5 µM each primer and 2.5 U *Taq* DNA polymerase (GIBCO BRL) in a Perkin Elmer 9600 with a fluorescent (AR-F, 5′–TCCAGAATCTGTTCCAGAGCGTGC–3′) and non-fluorescent (AR-R, 5′–GCTGTGAAGGTTGCTGTTCCTCAT–3′) primer for 30 cycles at 95 C° for 10 s, 63 °C for 5 s and 72 °C for 15 s. An initial denaturation was performed at 95 °C for 2 min, and a final extension at 72 °C for 7 min. PCR products (1.5 µl) were analysed on an ABI 373A Sequencer (Perkin Elmer/Applied Biosystems) with GENESCAN 672 software according to the manufacturer's specifications.

To examine methylation at the *AR* locus, 150 ng of DNA was digested in a 30-µl reaction volume with either 10 U of *Rsa*I alone or 10 U of *Rsa*I with 15 U *Hpa*II in buffer #1 (NEB) for 16 h at 37 °C (ref. 16). The enzymes were heat-inactivated for 10 min at 95 °C. Each digestion (3 µl) was amplified in a reaction mixture containing 20 mM Tris (pH 8.4), 50 mM KCl, 200 µM dNTPs, 0.5 µM each primer and 2.5 U *Taq* DNA polymerase (GIBCO BRL) in a Perkin Elmer 9600 with the AR-F and AR-R primers and PCR conditions as described above. A similar analysis at the *FMR1* locus was performed as described[23].

Sequence analysis. To determine *XIST* sequence, 50–200 ng of DNA from I-1, II-1 or II-3 (family I), mouse–human somatic-cell hybrid DNA derived from individual I-1 containing either an active or inactive X chromosome[20] and DNA from a series of control individuals were amplified with primers from the 5′ region of the *XIST* gene in a 25-µl reaction volume. The PCR reaction (15 µl) was purified with a QIAquick Spin PCR Purification Kit (Qiagen) and eluted in 50 µl of water; 8.5 µl of the purified fraction was sequenced with 3.2 pmol of either primer according to the conditions outlined by the ABI 373 Sequencing System (Perkin Elmer/Applied Biosystems). Sequence data were analysed with DNASTAR software.

***Hha*I restriction digest.** Fibroblast, lymphocyte or mouse–human somatic-cell hybrid DNA (50–200 ng) was amplified on a 9600 GeneAmp (Perkin Elmer) in a 25-µl reaction volume with G7R (5′–GAAGTTGTGACTC-CTGGTCT–3′) and G10R (5′–GAGAGATCTTCAGTCAGGAAG–3′) primers in a reaction mixture containing 20 mM Tris (pH 8.4), 50 mM KCl, 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM each primer and 2.5 U *Taq* DNA polymerase (GIBCO BRL) for 30 cycles at 94 °C for 30 s, 55 °C for 15 s and 72 °C for 45 s. An initial denaturation was performed at 95 °C for 2 min and a final extension at 72 °C for 7 min. *Hha*I (10 U) was added directly to the reaction volume and incubated at 37 °C for 30–60 min. The bands were resolved on a 2.5% ethidium-bromide–stained agarose gel.

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