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The relationship between physical and genetic distances at the *Hor1* and *Hor2* loci of barley estimated by two-colour fluorescent in situ hybridization

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Abstract The hordeins are the major class of storage proteins in barley. They are encoded by multigene families. The B- and C-hordein loci have been mapped physically to the distal end of chromosome 5 (1I) of cultivated barley by fluorescent in situ hybridization. Based on measurements of chromosomal distances between the two hordein loci, the relationship between genetic and physical distances has been estimated to be about 1 mega base pairs per centiMorgan. This is four times higher than the mean value for the barley genome as a whole and confirms the tendency to increased recombination in distal chromosome regions. The resolving power of two-colour FISH is discussed. It is concluded that the method is suitable for estimating the relationship between genetic and physical distances of regions of about 10 Mbp or larger.

Key words *Hordeum vulgare* · Physical and genetic chromosome maps · Recombination · In situ hybridization · Hordeins

Introduction

Information on the position of genes on a genetic map is necessary for estimating the feasibility of cloning a particular gene by "chromosome walking" (Arondel et al. 1992; Zhang et al. 1994). In many crop species, e.g. cultivated barley, *Hordeum vulgare* L., map-based cloning is difficult due to large genomes with high contents of repeated DNA-sequences. Thus, knowledge about the relationship between recombination estimates and physical distances in the chromosome region of interest is very important. In addition, this type of knowledge provides information on the distribution of recombination along chromosomes.

Several strategies are available for the integration of genetic and physical chromosome maps. A direct approach is to use genetically mapped RFLP probes for in situ hybridization to metaphase chromosomes, but the small size of most RFLP probes are at the limit of detection by in situ hybridization (Gustafson and Dillé 1992). Another approach is to use probes representing known-function genes which are present in sufficiently high copy number to produce in situ hybridization signals of satisfactory quality. In cultivated barley examples of such genes are the 5*s* rRNA genes, the α-amylase gene, and the B-hordein genes (Lehfer et al. 1993; Leitch and Heslop-Harrison 1993). In the present study the B-hordein locus, *Hor2*, the C-hordein locus, *Hor*1, and the telomeric repeat from *Arabidopsis thaliana* were mapped by fluorescent in situ hybridization (FISH) to chromosome 5 (1I). In addition, the physical distances between the three loci and the resolving power of two-colour FISH to metaphase and prometaphase chromosomes was examined by using various combinations of the probes. The two hordein loci flank the *Mla* region which contains genes for resistance to the powdery mildew fungus, *Erysiphe graminis* DC. ex Mérat f.sp. *hordei* Em. Marchal. This region was subject to chromosome walking attempts in order to isolate a resistance gene.

Materials and methods

Root-tip and chromosome preparations

Root-tips were obtained from seedlings of *H. vulgare* cvs Emir, Alexis, Igri and Montcalm. Meristematic cell divisions were partly synchronized by a hydroxyurea (HU) treatment as previously described (Pedersen and Linde-Laursen 1994). After the HU treatment the seedlings were rinsed in tap water followed by a 6-h incubation on moist filter paper, before pre-treatment with 4 μM of APM (Amiprophos methyl, kindly provided by MILES Inc., Kansas City, USA) for 2–3 h, with ice water overnight, or with APM for 2–3 h followed by ice water overnight (Pan et al. 1993).

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Chromosome preparations were either made by the squashing method using enzyme-treated root-tips as described by Anamthawat-Jónsson et al. (1993) or by the dropping method (Busch et al. 1994).

Probes and probe labelling

Three different probes were employed. The B-hordein probe consists of a 3.5-kb and a 1.2-kb *Eco*RI-fragment both originating from the genomic clone λhor 2–4 (Brandt et al. 1985). The C-hordein probe contains a 2.0-kb *Hin*dIII fragment from λhor 1–14 (Entwistle 1988). The telomere probe from *A. thaliana*, pAtT4, contains a 0.4-kb insert which consists of tandem repeats of the sequence 5′-TTTAGGG-3′ (Richards and Ausubel 1988). The hordein clones were kindly provided by A. Brandt, Copenhagen, and the telomere probe by F. M. Ausubel, Bostom.

Inserts were labelled with biotin or digoxigenin (DIG) either by nick translation (Gibco, BRL) or by random primed labelling using the BioPrime DNA-labelling system (Gibco, BRL). In the case of DIG labelling the nucleotide solution was modified to correspond to the inclusion of DIG-11-dUTP (Boehringer Mannheim).

In situ hybridization

The in situ hybridization procedure followed essentially the protocol described previously (Pedersen and Linde-Laursen 1994). However, a few steps were modified. The proteinase K treatment was in some instances substituted by a treatment with 7.5 μg/ml of pepsin (Sigma) in 0.01 N HCl at 37 °C for 10 min. For two-colour FISH detection the slides were first incubated with 5 μg/ml of FITC-avidin DCS (Vector). After washing, the slides were incubated with 5 μg/ml of biotinylated anti-avidin (Vector) and 0.25 μg/ml of anti-DIG (Boehringer Mannheim) followed by incubation in a solution containing 5 μg/ml of FITC-avidin DCS and 5 μg/ml of anti-mouse-Cy3 (Sigma).

Microscopy, photography, and measurement of chromosomal distances

The slides were examined with a Zeiss Photomicroscope III equipped with a 100 × Neofluar objective and filter sets for DAPI (487702), FITC (487709) and Cy3 (487715). Strong Cy3 signals were to some extent visible through the FITC filter set permitting observation of the two fluorochromes at the same time. Photographs were taken on Kodak Ektachrome P800/1600 professional film for colour slides or on Fujicolor 400 Super G film for colour prints. Chromosomal distances were calculated on the basis of slide projections to a magnification of approximately 26 000 × of somatic metaphases. The fraction lengths (FLs) (%) of the hybridization sites were calculated as the distance from the centromere to the hybridization signal relative to the total length of the chromosome arm. Standard deviations were estimated for all calculations.

Results

In situ hybridization with B- and C-hordein probes

In situ hybridization with the B-hordein probe hor 2–4 produced a clear signal at the distal end of the short arm of chromosome 5 (Fig. 1a, b). Occasionally, this was seen as a double-signal on each chromatid especially on prometaphase chromosomes (Fig. 1b). In situ hybridization with the C-hordein probe hor1-14 also produced a signal at the distal end of chromosome 5 (Fig. 1c, d), but the strength of the signal was weaker than that of hor 2-4. The frequency of detection of double signals with the hor1-14 probe was about 50% in the best slides.

Fig. 1a–i Fluorescent in situ hybridization (FISH) to somatic metaphase chromosomes of barley (2n = 14). In all cases scale bar = 5μM. **a** Using the B-hordein probe, hor2-4, labelled with biotin and detected with FITC-avidin after one round of amplification (two layers of FITC-avidin). **b** FISH to prometaphase chromosomes with hor 2-4. The hybridization signals are seen as double-spots on each chromatid (*arrows*) indicating a division of the B-hordein locus into two sub-loci. **c** FISH to metaphase chromosomes of barley using the C-hordein probe, hor 1-14, labelled with biotin and detected with FITC-avidin after one round of amplification (two layers of FITC-avidin) (*arrow*). Orange telomere signals produced by the telomere probe, pAtT4, detected with Cy3, are seen on some chromosomes. **d** FISH to metaphase chromosomes of barley using the C-hordein probe, hor 1-14, labelled with biotin and detected with FITC-avidin after one round of amplification (two layers of FITC-avidin) (*arrow*). **e** and **f** Two-colour FISH to metaphase chromosomes. Hor1-14 is labelled with biotin and detected with FITC after one round of amplification (*long arrows*) while hor 2-4 is labelled with digoxigenin (DIG) and detected with one layer of anti-DIG (mouse) and a second layer of the fluorochrome Cy3 conjugated to anti-mouse antibodies (*short arrows*). Both signals are visualized simultaneously by the filter combination for FITC (**e**), while the hor 2-4 signals are observed selectively using the filter combination for Cy3 (**f**). **g** Two-colour FISH to metaphase chromosomes using hor 1-14 (FITC) (*long arrow*) and the telomere probe, pAtT4 (Cy3) (*short arrow*). The orange telomere signals are seen on most chromosomes. **h** and **i** Two-colour FISH to metaphase chromosomes using hor 1-14 (Cy3) (*short arrow*), hor 2-4 (FITC) (*long arrow*) and pAtT4 (Cy3) (*short arrow*). Observation of both colours simultaneously (**h**) and the Cy3 signals only (**i**)

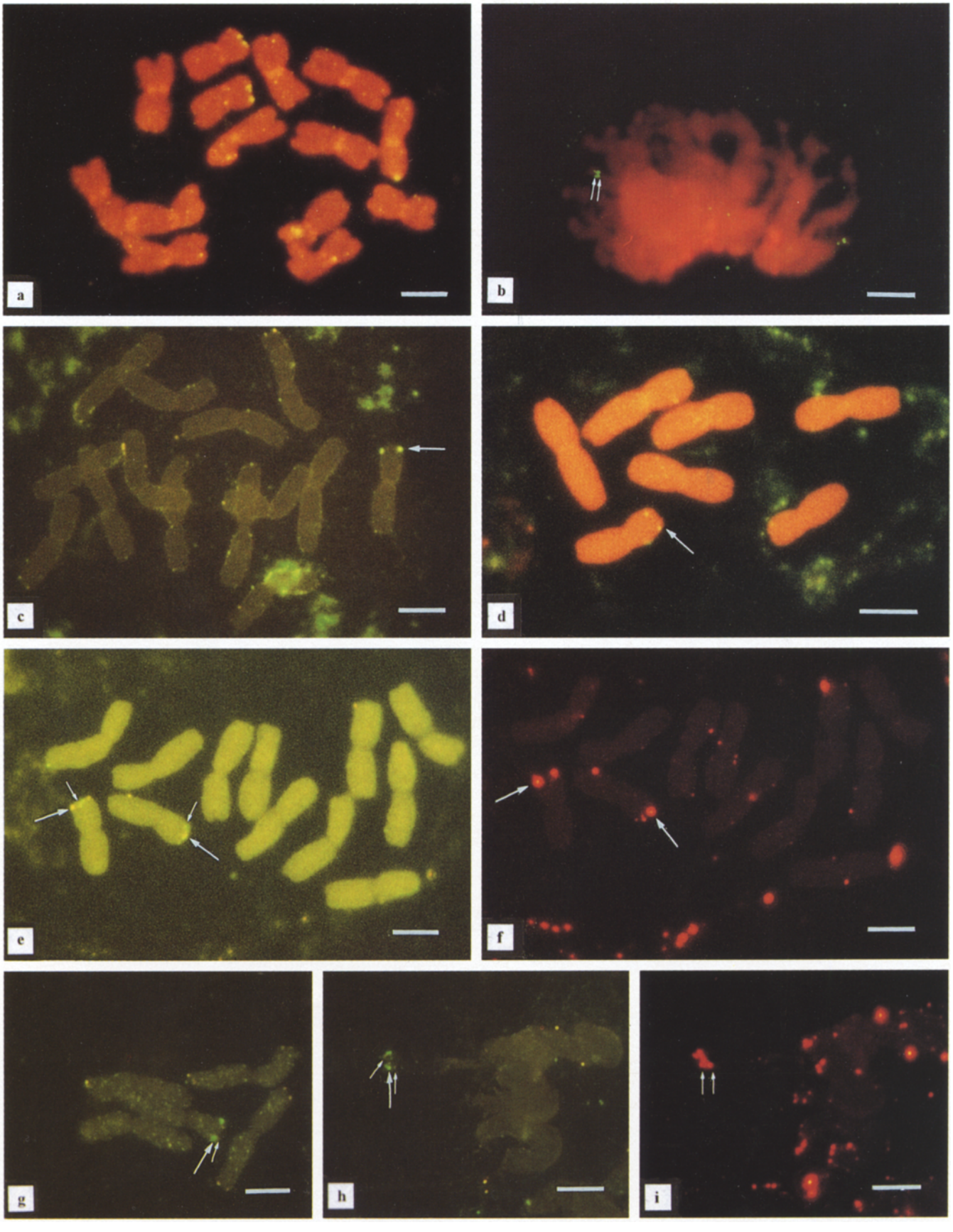
Two-colour fluorescent detection of the two hordein probes hybridized simultaneously to metaphases and prometaphases permitted a more detailed study of their physical location. On some metaphase chromosomes the two hordein probes showed an almost side-by-side localization, but usually the *Hor2* probe was located distal to the *Hor1* probe (Fig. 1e, f). A better physical separation of the two hordein loci was obtained on prometaphase than on metaphase chromosomes.

Two-colour FISH with combinations of B- and C-hordein probes and the telomere probe

The telomere probe pAtT4 labels the absolute end of all chromosomes. Metaphases observed after two-colour FISH using the hor 1-14 probe and the telomere probe showed a clear physical separation of the two loci (Fig. 1g). This also applied to combinations of the hor2-4 and telomere probes. However, the latter two probes showed an almost side-by-side localization on some metaphase chromosomes.

Physical mapping of B- and C-hordein loci

Two-colour FISH experiments with the three probes were carried out by labelling hor 1-14 and pAtT4 with DIG and hor2-4 with biotin. DIG was detected with Cy3 and biotin with FITC. The three probes could be observed and photographed simultaneously by using the filter combination 487709 for FITC (Fig. 1h, i). The opposite procedure of labelling and detection of the


a
b
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e
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i

Table 1 Fraction lengths of *Hor1* and *Hor2* loci in barley. Calculations are based on measurements in situ hybridization signals on 24 metaphase chromosomes with signals from the C-hordein probe, the B-hordein probe, and the telomere probe, pAtT4

Locus	Fraction length	SD
Hor1	89.1%	3.0%
Hor2	93.9%	2.7%
Distance between *Hor1* and *Hor2*	4.7%	2.5%

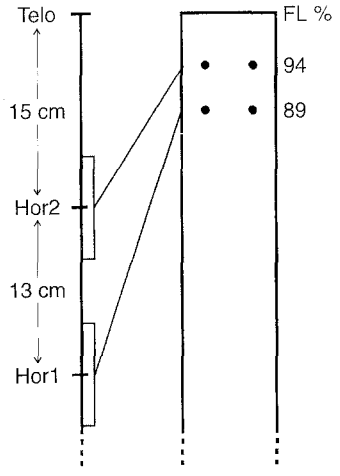

Fig. 2 Schematic presentation of the locations of the *Hor1* and *Hor2* loci on the distal part of the genetic map (in cM) (to the left) and the physical chromosome map (in FL%) (to the right) of the short arm of chromosome 5 (1H). The variations of the positions of the HOR loci on the genetic map are indicated

three probes gave the same result. Twenty-four metaphase chromosomes with signals from all three probes were measured. The fraction lengths (FLs) were calculated to be 89% for *Hor1* and 94% for *Hor2* (Table 1 and Fig. 2). The distance between the two loci was calculated to be 4.7% with a standard deviation of 2.5%. Although prometaphase chromosomes showed a clearer separation of the probes, they were not included in the calculations because of a clearly uneven condensation.

Discussion

The present study addresses three important aspects of gene mapping in barley: (1) the physical locations of the *Hor1* and *Hor2* loci on chromosome 5, (2) the relationship between the physical and genetic distances in the *Hor1-Hor2* region, and (3) the resolving power of two-colour FISH on metaphase- and prometaphase-chromosomes.

Physical location of B- and C-hordein loci

B-hordeins are encoded by a multigene family containing at least 15–30 members (Kreis et al. 1987). On the basis of the migration rates on SDS gels, B-hordeins are divided into three classes, B1, B2 and B3. B1 and B3 represent the major classes. Each class contains a number of polypeptides as shown by two-dimensional electrophoresis (Faulks et al. 1981). Sørensen (1989) analysed the physical organization of the *Hor2* locus using the pulsed-field gel electrophoresis (PFGE) technique. He mapped the hor2-4 probe to two different fragments of 160 and 200 kb indicating a division of *Hor2* into two sub-loci. A rare recombination event within the *Hor2* locus between the B1- and the B3-classes supports such a subdivision (Shewry et al. 1990; Kanazin et al. 1993). The localization of the B-hordein locus, *Hor2* as observed by in situ hybridization to the distal end of the short arm of chromosome 5 at 94% FL in the present study, is in good accordance with the localization reported by Lehfer et al. (1993). These authors also observed double signals on each chromatid as reported here. It is reasonable to conclude that the double signal reflects the physical separation of the two B-hordein subloci.

The C-hordeins can be separated into five polypeptide groups based on size. The groups can be further separated into at least ten types by two-dimensional analysis (Holder and Ingversen 1978). Southern-blot analysis has indicated that the *Hor1*-gene family comprises 6–8 members (Rasmussen and Brandt 1986). Siedler and Graner (1991) have constructed physical maps of the *Hor1* locus by PFGE. They showed that the maximal extent of the locus is 135 kb. Thus, it is less complexly organized than the *Hor2* locus. In agreement with this, only one locus was noticed in this study. The C-hordein locus, *Hor1*, was mapped at an FL value of 89%. The *Hor1* locus has not previously been mapped by in situ hybridization.

Calculation of the relationship between physical and genetic distances at the *HOR* loci

There is considerable variation in the genetic distances found between the *Hor1* and *Hor2* loci depending on the cross examined. The values reported have been as low as 4.6 cM and 6.9 cM (Shewry et al. 1978; Sozinov et al. 1979), and as high as 19 cM and 22 cM (Doll and Jensen 1986; Giese et al. 1994). When data from numerous crosses are merged, the mean distance between *Hor1* and *Hor2* is approximately 13 cM (Jensen 1993). This value is used in the following calculation of a general estimate of the relationship between the physical and genetic distances in this region. However, if instead the Mb/cM estimate has to be used in connection with map-based cloning, the genetic distance between *Hor1* and *Hor2* in that particular cross provides a better estimate for calculation of the Mb/cM ratio.

The physical distances in megabase-units are generally calculated from chromosomal distances assuming that the chromosomes are evenly condensed. The barley genome is estimated to have a size of 5360 Mb (Bennett

and Smith 1976). The size of the short arm of chromosome 5, which constitutes 5.1% of total genome length (Jensen and Linde-Laursen 1992), is thus about 270 Mb. We have measured the chromosomal distance between the *Hor1* and *Hor2* loci to be about 5 FL-units, which equals approximately 14 Mb. On this basis, the relationship between the physical and genetic distances in the *Hor1-Hor2* region is about 1 Mb/cM. However, due to variation in the estimation of the genetic distance in this region, and the uncertainty of the physical distance (Fig. 2), this is a very rough estimate. The *Hor2* locus has been mapped relative to a PCR-based telomeric marker with a distance of approximately 15 cM on the Steptoe × Morex map generated by NABGMP (Kilian, personal communication). Using a similar calculation as for the *Hor1-Hor2* region, a physical-genetic correlation of about 1 Mb/cM is also estimated for the telomeric region distal to the *Hor2* locus.

The total genetic length of the short arm of chromosome 5 is approximately 60–80 cM, which gives about 4 Mb/cM on an average. Künzel et al. (1994) have calculated values from about 50 Mb/cM in the centromeric region to 1.28 Mb/cM within the distal quarter of the short arm of chromosome 5. Their results, which are based on PCR with specific primers on micro-isolated translocation chromosomes with defined breakpoints, are in good agreement with the present results. A strongly reduced recombination in the inner half of the chromosome arms and an increased recombination in the distal region have previously been observed in barley (Linde-Laursen 1979, 1982; Laurie et al. 1993), wheat and rye (Lukaszewski 1992; Kota et al. 1993).

Resolving power of two-colour FISH

In situ hybridization with the three probes provides an indication of the resolving power of FISH to metaphase and prometaphase chromosomes. This study shows that in barley it will be difficult to determine the order of loci separated by less than 5–10 Mb, which corresponds to only a few FL-units on the metaphase chromosomes. Furthermore, there is quite a high uncertainty on measurements of such short distances on metaphase chromosomes. Leitch et al. (1994) indicate that the maximum resolution on cereal metaphase chromosomes is around 3 Mb, which is close to the limit of a few Mb of metaphase mapping on human chromosomes (Lawrence et al. 1990). Prometaphase chromosomes, which may be twice as long as, or longer than, metaphase chromosomes, may permit the spatial separation and ordering of physically closely linked clones, especially in the distal and most decondensed regions. However, since prometaphase chromosomes show an uneven condensation it is not possible to transform FL-units observed on them into Mb-units.

In conclusion, two-colour FISH to metaphase chromosomes is suitable for estimating the relationship between genetic and physical distances corresponding to about 10 Mb or more. This approach is, however, hampered by the limited number of RFLP probes which presently can be mapped to chromosomes by FISH. Thus, the method is especially suitable for giving a general overview of the distribution of recombination over larger chromosome regions. For map-based cloning the PFGE technique is necessary for estimating more accurately the relationship between genetic and physical distances in the region around the locus of interest flanked by the closest RFLP markers.

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