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Genetic Linkage among Cloned Genes of *Streptococcus mutans*

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Mapping vectors containing antibiotic resistance markers inserted adjacent to or within different cloned genes from *Streptococcus mutans* were used to determine the relative positions of these genes on the chromosome. The *gtfA*, *ftf*, and *scrB* genes were inserted into streptococcal mapping vector pVA891 adjacent to an Em^r gene, whereas the Em^r marker was inserted directly into the *gtfB* gene. These chimeric plasmids were transformed into *S. mutans* GS-5, selecting for Em^r transformants. To determine the positions of the cloned genes relative to each other, it was necessary to construct plasmids labeled with a different antibiotic resistance marker. Thus, a Tet^r gene was inserted adjacent to *gtfB* in the appropriate mapping vector and within the *ftf* and *scrB* genes with a mini-Mu transposon (Mu dT). The chimeric plasmids were transformed into the appropriate Em^r recipients, and the DNA from the resulting Em^r Tet^r transformants was used in linkage studies. Based on the cotransfer data, *gtfB* was not closely linked to *gtfA*, *ftf*, or *scrB*. However, *gtfA* cotransferred with *ftf* and *scrB* at frequencies of approximately 96 and 80%, respectively. The percent cotransfer of *ftf* and *scrB* was approximately 92. These data indicate that the three genes are clustered on the GS-5 chromosome, with *ftf* located between *gtfA* and *scrB*. Little, it any, linkage was observed between these genes and a variety of other random markers.

The production of glucosyltransferases by *Streptococcus mutans* enables these organisms to synthesize both soluble and insoluble glucans from sucrose (11). Although it is recognized that the synthesis of insoluble glucans enhances the colonization of tooth surfaces, the role of the soluble polymer and the number and specific role of each glucosyltransferase have not been clearly elucidated (4). At least two distinct glucosyltransferases appear to be involved in the synthesis of insoluble glucans (10, 14), one catalyzing the synthesis of primarily α-1,3-glucose linkages and the other catalyzing the synthesis of α-1,6-glucose linkages. It was suggested that soluble glucan acted as an autoprimer for the synthesis of insoluble glucan and that de novo synthesis of the former polymer was essential for the formation of the latter (7). Supporting this view was the finding that a dextranase specific for α-(1,6) linkages (soluble glucans) inhibited sucrose-dependent colonization (23). A *gtfA* gene catalyzing the synthesis of a low-molecular-weight glucan has also been described (16, 17), but its role in adherence is uncertain. In addition to glucosyltransferases *S. mutans* strains also produce fructosyltransferases (FTF), which synthesize both soluble and insoluble fructans from sucrose (4, 22), and sucrose-6-phosphate hydrolase, which is involved in the sucrose phosphotransferase system (3, 25).

The multitude of sucrose-metabolizing enzymes produced by most strains of *S. mutans* have added to the difficulty of isolating and characterizing those enzymes involved in the cariogenic process. One approach which should prove successful, however, has been the isolation of individual *S. mutans* genes by molecular cloning (9). In this regard, we recently initiated attempts to map cloned *S. mutans* genes by inserting antibiotic resistance markers adjacent to relevant genes on the chromosomes of these organisms. With this approach, the genetic location of *gtfA* (15) was indirectly determined by positive selection for an erythromycin resistance (Em^r) marker. The results showed that *gtfA* was cotransferable with a methionine marker but not with a number of other markers. The present investigation describes the linkage analysis of *gtfA* and the *S. mutans* genes *gtfB* (glucosyltransferase catalyzing the synthesis of primarily α-1,3-glucose linkages; 1), *ftf* (FTF; 21), and *scrB* (sucrose-6-phosphate hydrolase; 6), which recently have been cloned.

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MATERIALS AND METHODS

Bacterial strains, DNA preparation, and transformation. The bacterial strains and plasmids used in this study are listed in Table 1. Isolation of mutants, maintenance, and growth of cultures were as previously described (15). Chromosomal and plasmid DNA preparations, transformation procedures, and media used to select for transformants were all recently described (15). Em^r and tetracycline-resistant (Tet^r) transformants were scored on mitis salivarius agar or Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) containing 10 μg of erythromycin and/or 4 μg of tetracycline per ml.

Southern blot analysis. Restriction enzyme digestion, filling in with Klenow fragment of DNA polymerase I, and ligation were performed in accordance with the directions of the suppliers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.; International Biotechnologies, Inc., New Haven, Conn.). DNA fragments were separated by electrophoresis on 0.7% agarose gels, transferred to nitrocellulose paper (13), and hybridized with biotin-labeled probes under the conditions described by the supplier (Bethesda Research Laboratories).

Construction of hybrid plasmids. Details of the isolation of cloned *S. mutans* genes with the phage λ L47.1 vector system were described elsewhere (1). A portion of the *gtfB* gene was contained on a 1.6-kilobase (kb) *Bam*HI fragment, with a 1.8-kb Em^r gene replacing the 0.3-kb *Hin*dIII fragment (pTS61; 1). The Em^r gene was derived from streptococcal shuttle plasmid pVA891 (12). The FTF subclone, pSS7 (21), was digested with *Hin*dIII, and a 3.4-kb fragment containing the *ftf* gene was isolated by agarose gel electrophoresis and inserted into the *Hin*dIII site of plasmid pVA891 (pSS22e). The *scrB* gene was isolated on a 3.4-kb fragment from

TABLE 1. Bacterial strains and plasmids

Plasmid or strain	Description	Reference or source
Plasmids		
pUC8	Ap^r *E. coli*	5
pBGS8	Km^r *E. coli*	24
pAM148	Tet^r *E. coli*	D. B. Clewell
pVA891	Em^r	12
pSS7	*ftf* (5.4 kb)	21
pMH613	*scrB* (6.6 kb)	6
pLN1	pBGS8-pAM148 ligate	This study
pLN2	Tet^r	This study
pTS61	*gtfB* Em^r	1
pNP9	*gtfB* Tet^r	This study
pSS22e	*ftf* Em^r	This study
pDP22t	*ftf* Tet^r (Mu dT)	This study
pMH146e	*scrB* Em^r	This study
pDP146t	*scrB* Tet^r (Mu dT)	This study
pHK601e	*gtfA* Em^r	15
S. mutans GS-5 serotype c strains		
HK601e	GS-5(pHK601e)	This study
HK22e	GS-5(pSS22e)	This study
HK146e	GS-5(pMH146e)	This study
LN61e	GS-5 containing 3.4-kb *gtfb*	This study
GtfA/GtfB	HK601e(pNP9)	This study
Ftf/GtfB	HK22e(pNP9)	This study
ScrB/GtfB	HK146e(pNP9)	This study
GtfA/Ftf	HK601e(pDP22t)	This study
GtfA/ScrB	HK601e(pDP146t)	This study
Ftf/ScrB	HK22e(pDP146t)	This study

pMH613 (6) after digestion with *Sau*3A1. This fragment was subsequently inserted into the *Bam*HI site of pVA891 (pMH146e). Construction of pHK601e (*gtfA* Em^r) was described previously (15).

Plasmid pNP9 (Fig. 1) containing the *gtfB* fragment inserted adjacent to a Tet^r gene was constructed by initially ligating *Sma*I-digested pBGS8 (24) and *Hin*cII-digested pAM148 (Tet^r; D. B. Clewell, personal communication). The Tet^r gene in pAM148 is expressed in both gram-positive and gram-negative bacteria. The resulting hybrid plasmid (pLN1) was digested with *Bam*HI to remove the 5.4-kb fragment containing the Tet^r gene; this fragment was inserted into the *Bam*HI site of pUC8 to produce pLN2 (for convenient isolation of the Tet^r gene with a variety of different restriction enzymes). Subsequently, the Tet^r gene, isolated by agarose gel electrophoresis after *Bam*HI digestion of pLN2, was inserted into the *Bam*HI site of pBGS8, which also contained the 1.6-kb *Bam*HI *gtfB* fragment (1; filled in with Klenow fragment and inserted into the *Sma*I site of pBGS8). The pAM148 Tet^r gene was inserted into the *ftf* and *scrB* genes with the mini-Mu transposon Mu dT (H. K. Kuramitsu, unpublished data) as recently described (8). Plasmids pMH146e and pSS22e were the sources of the *scrB* and *ftf* genes, respectively. Mu dT contains the Tet^r gene, and transposition of Mu dT within either the *ftf* or the *scrB* cloned gene resulted in the subsequent introduction of the Tet^r marker into the GS-5 chromosomal genes following transformation. Plasmids pDP146t and pDP22t (Fig. 2) were linearized with *Eco*RI and *Cla*I, respectively, prior to transformation into *S. mutans*.

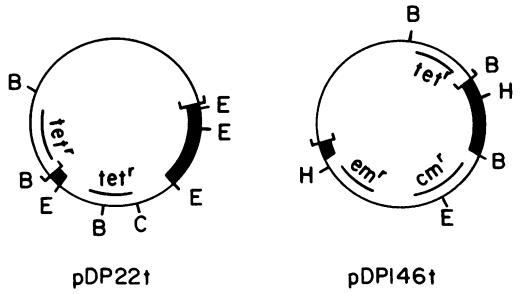

FIG. 2. Restriction maps of pDP22t (*ftf* Tet^r) and pDP146t (*scrB* Tet^r). The solid bars represent the *ftf* or *scrB* fragment; the thin lines within the brackets represent Mu dT. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII.

Each construct was confirmed by restriction enzyme analysis prior to and after transformation into *S. mutans*. The vectors used to construct hybrid plasmids replicate in *Escherichia coli* but not in streptococci. Thus, when these plasmids were transformed into strain GS-5, integration into the chromosome occurred by flanking homology (Fig. 1). Transformants were selected on media containing either erythromycin or tetracycline, and chromosomal DNA from these transformants was used to map the position of each GS-5 gene relative to each other and to a variety of auxo-

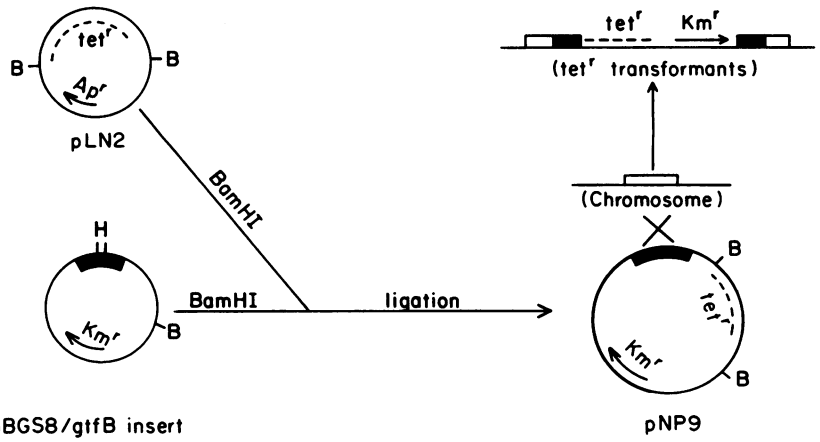

FIG. 1. Construction of pNP9 and its insertion into the homologous region of the GS-5 chromosome. The solid bars represent the 1.6-kb *gtfB* gene fragment. Endonuclease restriction sites for *Bam*HI and *Hin*dIII are indicated as B and H, respectively.

TABLE 2. Cotransfer of *gtfB* with *gtfA*, *ftf*, and *scr*B

DNA donor[a]	No. of the following transformants/0.1 ml			% Cotransfer[c]
	Em[r]	Tet[r]	Em[r] Tet[r][b]	
GtfA/GtfB	2,620	4,840	22	0.30
Ftf/GtfB	4,220	6,360	18	0.17
ScrB/GtfB	3,960	6,040	22	0.22

[a] The strain designations indicate the GS-5 genes containing the adjacent Em[r] and Tet[r] genes (e.g., strain GtfA/GtfB represents *gtfA* and *gtfB* adjacent to Em[r] and Tet[r], respectively). Strain GS-5 was transformed with 0.5 μg of each DNA per ml.

[b] The Em[r] Tet[r] transformants were selected by direct plating on Trypticase soy agar containing both antibiotics.

[c] Percent cotransfer = [Em[r] Tet[r]/(Em[r] + Tet[r])] × 100.

trophic markers. The percent cotransfer was defined as the number of double transformants divided by the number of single transformants, multiplied by 100.

RESULTS

Cotransfer of selected markers with *gtfB*, *ftf*, and *scrB*. To map the relative locations of the *S. mutans* genes, we constructed hybrid plasmids which would insert an Em[r] gene within or adjacent to each homologous region (*gtfB*, *ftf*, *scrB*) of the GS-5 chromosome. Thus, any marker linked to the GS-5 genes would also be linked to Em[r]. The hybrid plasmids, pSS22e and pMH146e, and the 3.4-kb *gtfB* fragment containing the Em[r] gene were transformed into strain GS-5, and limiting concentrations of DNA from the resulting Em[r] transformants were used to determine linkage between the Em[r] gene and a variety of random markers (dextranase [Dex], bacteriocin production, auxotrophs, sugar nonutilization). Of 15 different markers examined, only Dex and isoleucine-valine (Ilv) exhibited significant, although low, cotransfer with any of the inserted Em[r] genes (data not shown). These results showed that *gtfB* cotransferred with Dex at a frequency of approximately 6% and that *ftf* cotransferred with both Dex and Ilv at frequencies of approximately 6%. Despite these relatively low frequencies, cotransfer of the markers was independent of DNA concentration. In contrast, other markers (unlinked) exhibited cotransfer frequencies of less than 1% at limiting DNA concentrations. The *scrB* gene did not cotransfer with any of the random markers tested.

Cotransfer of *gtfB* with *gtfA*, *ftf*, and *scrB*. *S. mutans* HK601e (*gtfA* Em[r]), HK22e (*ftf* Em[r]), and HK146e (*scrB* Em[r]) were transformed with pNP9 (*gtfB* Tet[r]), selecting for Em[r] Tet[r] transformants. DNA (0.5 μg/ml) from these transformants was used to transform wild-type GS-5. Transformants were selected on mitis salivarius agar containing either erythromycin or tetracycline and replica plated onto mitis salivarius agar containing both antibiotics to determine the number of Em[r] Tet[r] transformants. The cotransfer frequencies ranged from 0.17 to 0.30% (Table 2), indicating that *gtfB* was not linked to *gtfA*, *ftf*, or *scrB*.

Linkage of *gtfA*, *ftf*, and *scrB*. To determine the linkage between these three genes, GS-5 strains HK601e and HK22e were transformed with pDP146t (*scrB* Tet[r]) DNA, and strain HK601e was also transformed with pDP22t (*ftf* Tet[r]) DNA. The DNA from the resulting transformants (Em[r] Tet[r]) was subjected to Southern blot analysis to verify the orientation and insertion of a single copy of each plasmid into the GS-5 chromosome (data not shown). Subsequently, this DNA was used to assay the frequency of cotransfer of Em[r] and Tet[r] (Table 3). The results indicated that *gtfA* cotransferred with *ftf* and *scrB* at frequencies of 95.8 and 80.1%, respectively. Furthermore, the frequency of cotransfer of *ftf* and *scrB* was 92.4%. As previously shown (Table 2), significant cotransfer was not observed for *gtfB* and *ftf*.

TABLE 3. Cotransfer of GS-5 Genes

DNA donor[a]	Em[r] Tet[r]/Em[r][b]	Em[r] Tet[r]/Tet[r][b]	% Cotransfer
GtfA/Ftf	117/121	132/139	95.8
GtfA/ScrB	99/127	127/155	80.1
Ftf /ScrB	66/69	80/89	92.4
Ftf/GtfB	1/100	0/100	0.5

[a] Strain GS-5 was transformed with 0.05 μg of each DNA. See Table 2, footnote *a*, for a description of the DNA donors.

[b] Transformants were selected on Trypticase soy agar containing the individual antibiotics and then replica plated to Trypticase soy agar containing both antibiotics.

Effect of DNA shearing on the cotransfer of GS-5 genes. The transfer of Em[r] and Tet[r] was moderately to highly sensitive to shearing (Table 4). Apparently, this sensitivity was independent of the association of each of these markers with a particular GS-5 gene. In addition, the sizes of the DNA fragments carrying the Em[r] (5.4 kb) and Tet[r] (13.2 kb) genes did not appear to be directly related to the sensitivity of these genes to shearing. As would be predicted from the linkage analysis, the GtfA/Ftf and Ftf/ScrB recombinants, which are closely linked, exhibited little loss of cotransfer activity after shearing. However, GtfA/ScrB, which cotransferred at a frequency of 72.8% with untreated DNA, lost almost half of this activity after shearing.

Effect of DNA concentration and phenotypic expression on the cotransfer of *gtfA* and *ftf*. The results obtained when the number of single and double transformants was determined as a function of GtfA/Ftf DNA concentration are shown in Fig. 3. The number of Em[r] and Tet[r] transformants were directly proportional to the limiting DNA concentrations. However, the percent cotransfer of these markers remained virtually the same irrespective of the concentration of the DNA. Both single and double markers appeared maximally after approximately 10 min of exposure of the cells to transforming DNA, although the Tet[r] marker did appear to be taken up by the cells at an approximately sevenfold faster rate (data not shown).

It was also of interest to examine the effect of time of phenotypic expression on the cotransfer of Em[r] and Tet[r] (Fig. 4). Tet[r] was fully expressed immediately after DNA uptake. In contrast, Em[r] was maximally expressed only after about 90 min of incubation. The number of Em[r] Tet[r] trans-

TABLE 4. Effect of shearing on the cotransfer of GS-5 genes

DNA donor[a]	% Residual transforming activity after shearing[b]		% Cotransfer		% Loss of activity
	Em[r]	Tet[r]	Unsheared	Sheared	
GtfA/Ftf	9.3	17.9	91.7	85.2	7.1
GtfA/ScrB	35.7	16.1	72.8	38.8	46.7
Ftf/ScrB	20.6	35.0	90.5	85.7	5.3

[a] Approximately 5 μg of each DNA in a volume of 0.1 ml was sheared by passage six times through a 0.5-in. (1.27-cm) 27.5-gauge needle equipped with a 1-ml syringe. Competent GS-5 cells were transformed with 0.05 μg of sheared or unsheared DNA per ml. See Table 2, footnote *a*, for a description of the DNA donors.

[b] Percent transformation with sheared DNA relative to unsheared DNA.

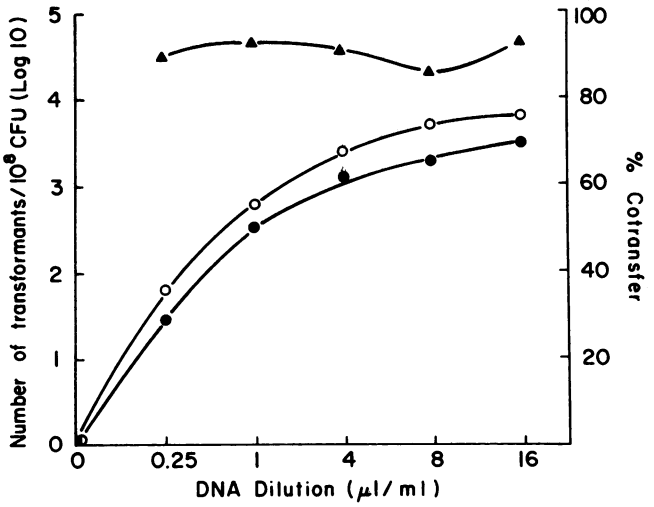

FIG. 3. Number of transformants as a function of GtfA/Ftf DNA concentration. Each microliter contained approximately 0.025 μg of DNA. Symbols: ●, Em^r (*gtfA*); ○, Tet^r (*ftf*); ▲, percent cotransfer.

formants, when plated directly, more or less followed the appearance of Em^r, also attaining maximum expression at 90 min. When the percent cotransfer of these markers was calculated and plotted against time of phenotypic expression, the ratio of double to single transformants only began to increase after 30 to 60 min. Again, maximum cotransfer was apparent after about 90 min. In this regard, it should be mentioned that the number of Em^r Tet^r transformants in this experiment was determined by direct plating on medium containing both antibiotics. For this reason, the cotransfer frequencies were lower than the values obtained by replica

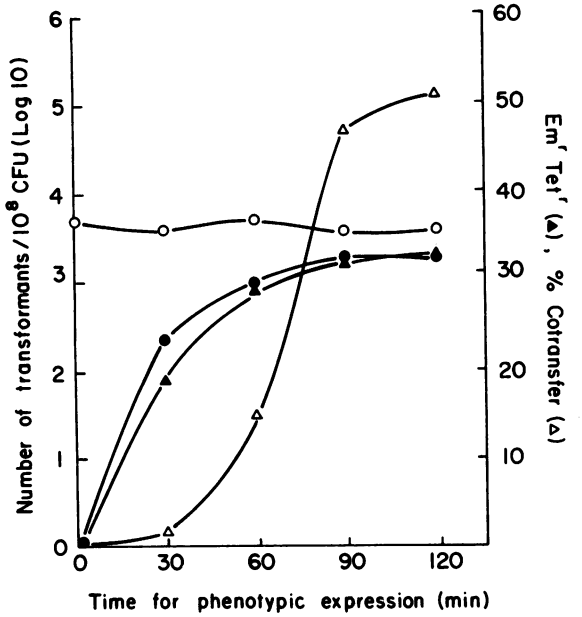

FIG. 4. Number of transformants as a function of time of phenotypic expression. GS-5 cells were exposed to 0.05 μg of GtfA/Ftf DNA for 15 min, DNase was added, and incubation was continued for the indicated times. Symbols: ●, Em^r (*gtfA*); ○, Tet^r (*ftf*); ▲, Em^r Tet^r; △, percent cotransfer.

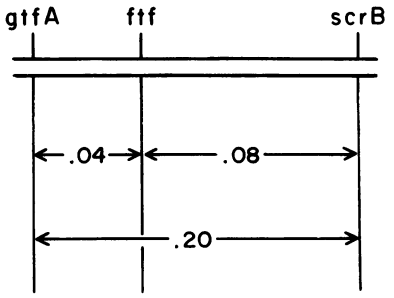

FIG. 5. Linkage map of GS-5 sucrose-metabolizing genes. Map distances were calculated from the values in Table 3 by the following formula: (1 − percent cotransfer)/100.

plating. Thus, the actual percent cotransfer of Em^r and Tet^r, as determined by replica plating, was approximately 95% even at 30 to 60 min of expression.

DISCUSSION

The clustering of genes of similar activities to facilitate the coordinate regulation of their expression has been widely demonstrated. Thus, a major objective of this investigation was to determine whether the genes coding for sucrose-metabolizing enzymes are localized randomly or clustered on the *S. mutans* chromosome. In previous studies, attempts were made to map the location of *gtfA* by determining its cotransfer frequency with a variety of auxotrophic markers together with bacteriocin and Dex production (15). These results, however, only showed that *gtfA* was linked to a methionine marker. Similar studies with *gtfB*, *ftf*, and *scrB* revealed that these genes were not linked to methionine, suggesting that they also were not linked to *gtfA*. Although *gtfB* and *ftf* cotransferred at a frequency of 6% with *dex*, these genes are obviously loosely linked, if at all. Interestingly, Burne et al. (2) found that *gtfA* was tightly linked to a Dex gene (*dexB*) on the GS-5 chromosome. However, it is not clear whether a single *dex* gene is present on the *S. mutans* chromosome.

The results of the present experiments showed that *gtfA*, *ftf*, and *scrB* are closely linked to each other but not to *gtfB*. The frequency of cotransfer data (Table 3) indicated that *ftf* lies between *gtfA* and *scrB* and is closer to *gtfA* than to *scrB* (Fig. 5). Chromosomal breakage during DNA isolation could account for the greater-than-expected map distance between *gtfA* and *scrB*. The relative sensitivity of the cotransfer of these genes to shearing supports this conclusion.

It would also be of interest to determine whether other *S. mutans* cariogenic genes are close to the *gtfA-ftf-scrB* gene cluster demonstrated in this study. In this regard, Russell et al. (18, 20) have isolated a glucan-binding protein that exhibits FTF activity. In contrast, the FTF derived from the pSS22e subclone in this study did not bind insoluble glucan (21). Since the *gbp* gene has been recently cloned (19), it should now be possible to determine the position of this gene relative to *ftf* and other related genes.12;5q

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