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Bipolar Localization of the Replication Origin Regions of Chromosomes in Vegetative and Sporulating Cells of B. subtilis

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Summary

To address the issue of chromosome movement in bacteria, we took advantage of a method recently devised by Straight et al. (1996) and Robinett et al. (1996) for visualizing specific chromosomal sites during mitosis in budding yeast and in Chinese Hamster ovary cells. The method is based on the insertion into the chromosome of Bacillus subtilis of a copy of the lacO cassette consisting of multiple (256) tandem copies of the operator for the E. coli lactose operon (lacO) and the use of a fusion of the green fluorescent protein (GFP) from Aequorea victoria to the lactose operon repressor LacI. Binding of GFP-LacI to the tandem operators causes the hybrid repressor molecules to cluster in a small spot that can be visualized by fluorescence microscopy.

Our strategy for visualizing chromosome segregation in bacteria was to insert a copy of the lacO cassette either near the replication origin or near the terminus of the chromosome of Bacillus subtilis. This spore-forming bacterium was advantageous for use in these studies because previous genetic experiments had led to the inference that at the start of the process of spore formation, when the sporangium contains two newly formed chromosomes, the replication origins become attached near opposite poles of the cell (Wu and Errington, 1994). We asked whether we could directly visualize the attachment of the replication origins at the start of sporulation. We also asked whether polar attachment was a normal feature of the cell cycle during binary fission. Here we report that the origins, but not the termini, of the two sporangial chromosomes do indeed localize near the ends of the sporangium. Moreover, we detect a population of cells at an early stage of the cell cycle in which the replication origin is preferentially located near the cell poles, a finding that indicates the existence of a mitotic-like apparatus that is responsible for moving the replication origin regions of newly duplicated chromosomes toward opposite ends of the cell.
Results and Discussion

Bipolar Localization of the Replication Origins during Sporulation

We first sought to visualize the replication origin and terminus in cells undergoing sporulation because the results of previous genetic studies had suggested that the region of the chromosome proximal to the origin is sequestered near the cell pole (Wu and Errington, 1994). Sporulating cells undergo a modified form of cell division in which a septum is formed at an extreme polar position to create a small forespore cell and a large mother cell. Unlike the process of binary fission in which DNA segregation precedes cytokinesis, the sporulation septum is formed prior to translocation of a complete chromosome into the forespore. Initially after polar septation, only a small portion of the chromosome is trapped in the forespore; the remainder is subsequently transported across the septum into the small sporangial compartment by a conjugation-like protein called SpoIIIE (Wu et al., 1995). Mutants defective in the spoIIIE gene are blocked in DNA translocation and hence their forespore compartment contains only about one-third of the normal complement of DNA (Wu and Errington, 1994; Sharpe and Errington, 1995). That this one-third corresponds to the origin-proximal region of the chromosome is inferred from the following chromosomal position effect on the expression of genes under the control of the forespore-specific transcription factor $\sigma^c$: in sporulating cells of a spoIIIE mutant, $\sigma^c$-controlled genes located proximal to the replication origin are actively transcribed whereas genes located distal to the origin are transcriptionally silent (Sun et al., 1991). This has been interpreted (Wu and Errington, 1994) to indicate that the replication origins are attached near the cell poles at the start of sporulation and that, in the absence of DNA translocation, only origin-proximal DNA is present in the compartment in which $\sigma^c$ is active.

To observe the origin and the terminus in sporulating cells, we engineered the synthesis of the GFP-LacI fusion protein in sporulating cells using a promoter ($spoVG$) that is active at the onset of sporulation (Zuber and Losick, 1983). The GFP-LacI construct was then introduced into cells that contained the operator cassette located near the replication origin region of the chromosome (at $amyE$ [25'] or $spo0J$ [359']) or near the terminus ($cgeD$ [181']).

GFP-LacI-producing bacteria that harbored the lacO cassette near the replication origin characteristically exhibited two green fluorescent dots near opposite poles of the cell (Figures 1A and 1E). Due to the low level of the fluorescent signal and a moderate level (30%–40%) of sporulation in our engineered strain, only a minority of the cells exhibited green dots (~11%). In other work involving the use of a cooled charge coupled device (CCD) camera, a higher proportion of sporangia exhibiting fluorescence was observed (~30%). Of those sporangia in which green dots were observed, a substantial proportion (61%, out of a total of 95 cells examined) exhibited a bipolar pattern of fluorescence (in many of the remaining sporangia only a single polarly localized dot was seen). When the gfp-lacI fusion was introduced into bacteria lacking the operator cassette, no green dots were observed above a uniform background of low level fluorescence.

Bacteria exhibiting green dots were mostly observed at an early stage of development, prior to the formation of the polar septum (Figure 1B). Fluorescent sporangia were harder to detect at later stages of development, although some postseptation sporangia were observed that exhibited a bipolar pattern of fluorescence (Figure 2B). Yet later, at the stage at which the forespore is wholly engulfed by the mother cell, the origins no longer appeared to be attached near the poles of either compartment (Figure 2C).

In contrast to the results obtained with the operator cassette near the origin, GFP-LacI-producing bacteria harboring the lacO cassette near the terminus of replication exhibited one or two green dots that were characteristic-ly located approximately in the middle of the cell (Figure 1C and 1G). For sporangia in which only one dot was evident, the two chromosomes may not have fully completed replication. Alternatively, these sporangia may contain two fully duplicated chromosomes but the termini may be in such close proximity that they could not be resolved by fluorescence microscopy. Once again, fluorescence was principally observed in sporangia at an early stage of development (Figure 1D). Among cells with a signal (11% of the total), a high proportion (74% out of a total of 149 cells) exhibited green dots in the middle region of the cells, and rarely, if ever, near both poles.

In summary, our observations are consistent with the idea depicted in Figure 3A that at the start of sporulation, chromosomes are oriented with their origins near opposite poles and their termini in close proximity to each other near the middle of the cell (i). Next, after polar septation (ii), the origin-distal region of the forespore chromosome is translocated into the small sporangial compartment (iii). Finally, after engulfment of the forespore by the mother cell, the replication origin ceases to be attached near either end of the sporangium (iv).

Polyplody in a Partition Mutant

Recent genetic experiments have implicated the spo0J gene in the anchoring of the replication origin region to the sporangial poles (Sharpe and Errington, 1996). Mutation of spo0J, which is related to the family of partition genes that are required for proper plasmid segregation in Gram-negative bacteria (Hiraga, 1992), causes a defect in chromosome segregation during growth and a block at the onset of sporulation (Ireton et al., 1994). This sporulation block can be overcome by a second mutation in soj (for suppressor of spo0J), which is also related to partition genes. Thus, a soj spo0J double mutant is capable of sporulating (Ireton et al., 1994). Interestingly, however, the strict chromosome position effect observed in a spoIIIE DNA translocase mutant is not observed in the presence of additional mutations in soj and spo0J. Thus, in a soj spo0J spoIIIE triple mutant, a low level of expression of $\sigma^c$-controlled genes located distal to the replication origin can be observed (Sharpe and Errington, 1996). These observations have been interpreted to indicate that spo0J is involved in polar attachment of the replication origins and that, in its absence, the chromosomes are not oriented and random.
Figure 1. Fluorescence Micrographs of Sporulating and Vegetative Cells with the Operator Cassette Near the Origin or the Terminus

The operator cassette is near the origin (amyE) in (A), (B), (E), (F), (I), (J), (M), (N), (Q), (R), (U), and (V) and near the terminus (cgeD) in (C), (D), (G), (H), (K), (L), (O), (P), (S), (T), (W), and (X). Arrows indicate the location of dots from GFP-LacI. (A, B, and E) Sporangia (strain AT16) exhibiting bipolar dots from the origin region (green in [A] and [E]) and a predivisional pattern of unsegregated chromosomal DNA (blue in [B], corresponding to sporangium in [A]). (C, D and G) Sporangia (strain AT18) with one or two dots from the terminus region and a predivisional pattern of chromosomal DNA ([C] and [D] show the same field of cells). (F, H±L) Multiple dots in soj spo0J mutant sporangia, with the operator cassette near the origin (F, I–J; strain AT19) or near the terminus (H, K–L; strain AT20). Note the gap between the forespore and mother cell chromosome as indicated by open arrowheads in (J) and (L). (M) Bipolar pattern of dots from the origin region in a pair of vegetative cells (strain CW437), with separated nucleoids in each (N). (O) Single dot from the terminus region in the middle of a vegetative cell (strain CW438), with nucleoids in the process of separating, seen in (P). (Q–X) Immunofluorescence of vegetative cells stained with anti-GFP antibodies (orange), with lectin to stain the cell wall (green), and with DAPI to visualize DNA (blue). (Q and R) Bipolar pattern of dots from the origin region (strain CW437) seen early in the cell cycle, prior to chromosome segregation. Line indicates location of a lectin-stained septum, not clearly seen in photograph. (U and V) Bipolar pattern of origin region dots (strain CW437) later in the cell cycle when the chromosomes have separated into two nucleoids. (S and T) Single dot from the terminus in a cell (strain CW438) in which the nucleoids have not yet fully separated. (W and X) Two dots from the terminus (strain CW438) seen in adjacent cells in which the nucleoids have begun to separate (left-hand cell) or have fully separated (right-hand cell). The scale bar in (A), which represents 2 μm, pertains to (A)–(P), whereas the scale bar in (Q), which also represents 2 μm, pertains to (Q)–(X).
regions of the chromosome become trapped in the forespore when the polar septum is formed. Indeed, consistent with the idea that Spo0J could be involved in sequestering the replication origin near the poles, other work (D. C.-H. L., P. Levin, A. G., unpublished data) has shown that Spo0J exhibits a bipolar distribution during sporulation (as well as during growth; see below).

To investigate the role of these partition genes in the polar attachment of the origin region during sporulation, we introduced the gfp-lacI construct and the lac operator cassette into a soj spo0J double mutant. Strikingly, multiple dots were observed when the operator cassette was located either near the origin or near the terminus (Figures 1F, 1H, 1I, and 1K). Among sporangia exhibiting a fluorescent signal, 53% (out of a total of 156 cells) had more than two dots. Moreover, sporangia were observed with up to eight dots when the cassette was located near the origin and up to four dots when the cassette was at the terminus. Because multiple origins and multiple termini were observed, some of the soj spo0J mutant sporangia must contain more than two complete copies of the chromosome. This is also evident from the size of the mutant cells: those cells with multiple dots were significantly larger than other cells (for example, compare the sporangium in Figure 1F with that in Figure 1E).

Most of the mutant sporangia exhibiting a fluorescent signal were at the predivisional stage of sporulation, but some sporangia with multiple chromosomes were observed that had appeared to reach the stage of polar division. These could be recognized by means of DAPI staining, which revealed a separated region of DNA, indicative of the presence of a forespore (Setlow et al., 1991; Resnekov et al., 1996) (Figures 1J and 1L). The existence of postseptation sporangia with multiple chromosomes suggests a possible alternative explanation for the absence of a strict chromosomal position effect in a soj spo0J spoIIIE triple mutant: polar septation in a sporangium containing multiple chromosomes could result in the trapping of random chromosomal regions within the newly formed forespore due to the presence of extra copies of the chromosome in the sporangium (Figure 3B). According to this view, Spo0J need not be responsible for anchoring the origin region to the poles. Instead, it may serve to coordinate cell division with chromosome copy number in cells entering sporulation so that normally only two chromosomes are present in cells undergoing polar division. Alternatively, Spo0J could be involved both in chromosome anchoring as well as in coordinating chromosome copy number with cell division.

Polar Localization of the Origin during Vegetative Growth

Next, we asked whether polar attachment of the replication origin region was unique to cells entering sporulation or a general feature of chromosome segregation.
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during asymmetric and symmetric cell division. To visualize the origin and terminus in growing cells, we engineered the expression of gfp-lacI in vegetative cells by placing the gene (spo0H) for $^{\alpha}$ under the control of the xylose-inducible promoter (Dubnau et al., 1988; Gartner et al., 1988). Thus, in such cells, xylose would enhance the levels of $^{\alpha}$ during growth and, hence, stimulate transcription of gfp-lacI from the $^{\alpha}$-controlled spo0VG promoter. Nevertheless, only a low level of green fluorescence was observed in such engineered cells.

To increase the sensitivity at which GFP-LaCl could be detected in growing cells, we turned to immunofluorescence using antibodies directed against GFP, an effective strategy that enabled us to observe signals for the GFP-LaCl fusion in over 40% of the cells. In the following experiments, GFP-LaCl appeared as orange dots whereas the cell wall and septa were stained green by the use of a lectin. Finally, nucleoids were visualized by the use of DAPI. The use of DAPI enabled us to distinguish cells at early stages of the cell cycle in which the nucleoid appears as a single filament along the length of the cell (Figures 1R and 1T) from cells at late stages of the cycle when the chromosomes have segregated into two distinct condensed nucleoids (Figures 1V and 1X).

First, we consider cells in exponential growth phase harboring the operator cassette near the origin. Among cells for which a signal could be seen, most (75%, out of a total of 140 cells) exhibited only a single dot, which was often located near a cell pole. The significance of such one-dot cells was difficult to interpret. They could represent cells in which the origin had not replicated or in which newly formed origins had not yet begun to segregate from each other. Alternatively, such cells could have contained two well-separated origins but, because of the low level of signal detection during growth, a second origin located out of the plane of focus might not have been detected. More informative was the case of cells exhibiting two dots (25%, out of a total of 140 cells). Among such cases, cells were often observed to have a dot close to each end. This bipolar pattern of dots was most prevalent at early stages of the cell cycle, when the chromosomes had not yet segregated (Figures 1Q and 1R), but could also be observed at later stages when the chromosomes had separated into two distinct nucleoids (Figures 1M, 1N, 1U, and 1V).

A similar pattern of bipolar localization was observed in experiments designed to visualize Spo0J in growing cells, reinforcing the view that Spo0J binds to the replication origin region and providing independent evidence that chromosomes become oriented with their origins toward the poles (D. C.-H. L. et al., unpublished data). Recently, the partition homologs ParA and ParB of the gram-negative bacterium Caulobacter crescentus have also been found to exhibit a bipolar pattern of localization, an indication that the orientation of the chromosomes with the origin toward the poles of the cell could be a general feature of chromosome segregation in prokaryotes (Moh and Gofer, 1997).

Next, we consider growing cells harboring the operator cassette near the terminus. Such cells generally displayed one or two dots located approximately in the middle of the cell (Figures 1O, 1S, and 1W). Although cells exhibiting two dots were infrequent, a bipolar localization pattern of termini tagged with the operator cassette was rarely if ever observed. Thus, our results indicate the existence of a population of growing cells in which newly formed origins, but not termini, are sequestered at or near opposite ends of the cell.

The above experiments were carried out with cells that were grown in a rich medium, conditions of rapid growth in which multiple replication forks are to be expected. When the generation time is shorter than the time required for duplication of a chromosome, a second round of replication is initiated before the first round of replication is complete (Cooper, 1991). Despite this expectation, we were not able to observe more than two dots in cells in which the origin was tagged with the lacO cassette. Thus, if multiple replication forks were present in rich medium, we were not able to detect them under our conditions. However, using a newly constructed strain (see below) that exhibits brighter fluorescence, we observed more than two dots in a bipolar growing cells. In light of this issue, we also carried out experiments with cells grown in a minimal medium (with glucose as the sole carbon source) in which no more than two replication forks were expected to be present. In minimal-medium grown cells with the lacO cassette at the origin, we detected cells with two dots (data not shown). Among such cells were those in which the dots were located in a bipolar fashion, similar to that observed for cells grown in rich medium.

Finally, to improve the efficiency with which operator-bound GFP-LaCl could be detected in growing cells, we created an additional fusion in which gfp-lacI was joined to a strong vegetative promoter (veg; Moran et al., 1982). Minimal-medium grown cells having the P_{veg}-gfp-lacI fusion exhibited much brighter signals than in the experiments described above (data not shown). Moreover, a high proportion (>90%) of the cells exhibited detectable fluorescence. As before, among cells harboring the P_{veg}-gfp-lacI fusion, a subpopulation could readily be observed that exhibited a bipolar pattern of dots when the operator cassette was at the origin but not when it was at the terminus (data not shown).

A Model for Chromosome Segregation

Our results suggest the following model for chromosome segregation during binary fission. As depicted in Figure 3Ci, the newborn cell initially contains a single chromosome and hence a single replication origin region. Next, after replication commences, the two newly formed origins are drawn toward opposite ends of the cell, eventually resulting in a cell (Figure 3Cii) with two complete chromosomes, each anchored near a cell pole from a site (centromere) located in the vicinity of its replication origin. At this stage, the two chromosomes are spread across the length of the cell in the form of a single filament, similar to that observed early in sporulation. Cells (Figure 3Cii) with a bipolar arrangement of replication origin regions could arise in one of two ways. One possibility is that before the initiation of replication, the origin region is not attached near either pole. Only after replication commences do the origins migrate toward the poles. Alternatively, the origin region
in a newborn cell could be attached near a pole prior to replication. Then after replication ensues, one of the two newly formed origin regions could be transported toward the opposite end of the cell. Our data do not allow us to distinguish between these possibilities decisively.

In the next stage of the cell cycle (Figure 3Ciii), the two chromosomes separate from each other, moving to opposite ends of the cells. A simple way in which this could occur would be by condensation of the chromosomes, each anchored near an opposite end of the cell. This condensation could be mediated by a protein similar to members of the SMC family of eukaryotic chromosome condensation proteins, homologs of which are known to exist in bacteria (Hirano et al., 1995). Finally, after each chromosome has coalesced into a distinct nucleoid, cytokinesis (Figure 3Civ) occurs by the formation of a septum in the gap between the separated chromosomes. If these ideas are correct, then a principal challenge for future investigations will be to define the nature of the centromere and the mitotic-like apparatus that effects centromere movement and chromosome segregation.

Experimental Procedures

Strain Constructions

To express the gfp-lacI fusion of Straight et al. (1996) in B. subtilis, the fusion was placed under the control of the spoVG promoter by being joined in-frame at the third codon of the spoVG open reading frame (ORF). DNA from the spoVG promoter region was obtained by PCR amplification using oligonucleotides SDMOL1 (5'-GCTGGCG GAAAGGGGATGTTG-3'), which corresponds to a vector sequence near the site of insertion of a spoVG-containing HindIII fragment in the HindIII site of pBSKS±, and AT03 (5'-TCTAGGATCCCATCGAT GTAATCCAGATGTTGACG-3'), which created adjacent Clal and BamHI restriction sites after the third codon of the spoVG ORF. The PCR product was digested with EcoRI and BamHI and ligated to similarly digested pDG795 to create pAT10. Plasmid pDG795 is an MLS-resistance gene-containing vector for creating insertions at the Thr locus on the B. subtilis chromosome (a gift of P. Stragier).

Next, the gfp-lacI gene fusion was amplified using pAFS78 (Straight et al., 1996) with oligonucleotides AT01 (5'--AAAAAGATC TGATGAATGTGAAAGG-3'), which created a BgIII site downstream of the open reading frame, and AT04 (5'--ATAGCATCGATGAGTAAA), which created a Clal restriction site 5' of the ATG start codon. The resulting PCR product, digested with BgIII and Clal, was ligated to BamHI-Clal-digested pAT10, yielding pAT11. This created an extended ORF in which three codons of spoVG were joined via a two codon linker to the gfp-lacI gene fusion. The plasmid was linearized by digestion with XhoI and used to transform wild-type, prototrophic B. subtilis strain PY79 (Youngman et al., 1984), followed by selection for MLS resistance (5 µg/ml). This yielded strain AT15, which was confirmed to be auxotrophic for threonine.

To insert the lacO-containing plasmid pAFS52 (Straight et al., 1996), into the chromosome near the origin (25'), we introduced into the chromosome at amyE a segment of DNA corresponding to the yeast pRE1 gene that was present in pAFS52. This 1.4 kb segment of pRE1 was introduced into the focus on the chromosome by the ampE-integrating vector pDG364 to create strain AT10. Next, a kanamycin resistance gene contained on a 1.9 kb BamHI-Sall fragment from pER82 (Drks et al., 1994) was introduced into pAFS52 to create pAT7. Strain AT10 was transformed with pAT7 following selection for kanamycin resistance (5 µg/ml). The resulting strain was then transformed with chromosomal DNA from AT15 following selection for MLS resistance (5 µg/ml), yielding AT16 which had both the lacO cassette at amyE (25') and the spoVG-gfp-lacI fusion. To generate a spo0J mutant derivative of AT16, the strain was transformed with chromosomal DNA from AG1505 (Ireton et al., 1994) followed by selection for spectinomycin resistance (100 µg/ml), which yielded strain AT20.

To express the spoVG-gfp-lacI fusion at higher levels during vegetative growth, an inducible spo0H fusion was made. A 550 bp Smal-EcoRI fragment from pSG20H (Fort and Errington, 1985) containing part of spo0H, with ends rendered flush with DNA Polymerase I Klenow fragment, was cloned into a Sall-digested pDAG6−1, yielding pCW75. Plasmid pDAG6−1 has a xylose-inducible promoter containing HindIII fragment from pDG1832 (a gift of P. Stragier) in the HindIII site of pJL74 (LeDeaux and Grossman, 1995). Plasmid pCW75 was inserted into the chromosome by a Campbell-like recombination at spo0H by transforming PY79 following selection for spectinomycin resistance (100 µg/ml), yielding CW436. Strains AT16, AT18, and AT25 were transformed with the chromosomal DNA of CW436 following selection for spectinomycin resistance (100 µg/ml) resulting in strains CW437, CW438, and AT29, respectively.

Growth Conditions

For vegetative growth, cells were grown overnight in Luria-Bertani (LB) medium at 25°C and then diluted 1:100 in either LB or S7 defined minimal medium (Cuatrecasas et al., 1989) grown at 30°C to an OD600 of 0.6. For sporulation, cultures were grown either in Difco sporulation medium (DSM) overnight at 25°C or on DSM plates overnight at 30°C (Nicholson and Setlow, 1990). The signal was brighter when cells were grown on plates. The number of copies of the lacO cassette in strains AT18 and AT29 was increased by growing the strains on higher concentrations of chloramphenicol, which also resulted in a brighter signal.

Microscopy and Photography

Immunofluorescence was carried out as previously described (Harry et al., 1995; Pogliano et al., 1995), with the following modifications: the fixation used contained 0.06% glutaraldehyde (v/v) in 16% paraformaldehyde (v/v), and the cells were treated with saponin for 3 min. The cells were not treated with methanol or acetone. The polyclonal rabbit anti-GFP antibodies (Clonetech) were used at 1:4000 dilution, and the incubation time was 1 hr at room temperature. Anti-rabbit Cy3 secondary antibodies were used at a 1:200 dilution and incubated for 1 hr at room temperature, with wheat germ agglutinin-fluorescein (10 ng/ml) (Molecular probes) and 1:6 Dilaminofluorene-2-phenylenedione (DAP) (2 µg/ml) (Sigma). Photomicrographs were taken as previously described (Harry et al., 1995; Pogliano et al., 1995), with the exception of the use of Fujichrome Provia400 film. Images were processed as previously described (Resnekov et al., 1996). For scoring, photomicrographs were taken using a Photometrics SenSys1400 Cooled CCD camera and a PowereMacG500 with IPLab Spectrum image processing software (version 2.5.6).

For observation of GFP signals, cells were resuspended in phosphate-buffered saline (pH 7.4) or water and placed on microscope slides. For better DNA staining, cells were lightly fixed as described for immunofluorescence above. The fix did not appear to affect the GFP signal, as similar results were observed without fixation. DAPI
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was added to a final concentration of 2 μg/ml. Color photomicro-

graphs were taken as previously described (Webb et al., 1995), with
the exception of the use of Kodak Ektachrome 400X. Black and white
photomicrographs were taken with the Photometrics Scion 1400
camera and a PowerMac9500 with IPLab Spectrum image pro-
cessing software (version 2.5.6). Images were processed as pre-
viously described (Resnekov et al., 1996).

Acknowledgments

We thank S. Meyer for plasmid pSM79, D. Garson for plasmid
pDAG8-1, P. Stragier for plasmids pDG795 and pDG1832, and O.
Resnekov for invaluable discussions and help with immunofluores-
cence experiments. C. D. W. is a predoctoral fellow of the National
Science Foundation. This work was supported by NIH grants
GM38035 to A. W. and GM18568 to R. L.

Received December 9, 1996; revised January 22, 1997.

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