Pure Yeast RNA Polymerase B (II) Initiates Transcription at Specific Points on Supercoiled Yeast DNA*

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Pure yeast RNA polymerase B (II) can selectively initiate abortive transcription on a supercoiled recombinant plasmid DNA carrying yeast DNA in the presence of low concentrations of ribonucleoside triphosphates and Mn2+. Five major products ranging between 60 and 150 nucleotides were characterized by hybridization. Three of them originate from the vector pBR322 and two from the yeast DNA insert. Based on a RNA primer extension reaction with recombinant M13 DNAs as template, a method allowing the mapping of the short abortive RNA products has been developed. An initiation site within the yeast DNA insert has thus precisely been mapped. The DNA sequence in this region was determined and showed several relevant features. The in vitro initiation site is preceded by a potential TATA box at −40 base pairs and at −105 by the sequence GTTAAATCT similar to the consensus sequence GATAATCT usually found around 80 base pairs upstream from the cap site. Large blocks of alternated purine pyrimidine residues are found in this region as for several known yeast promoters. The 5' end of the RNA initiated from this site contains several potential signals for the initiation of translation.

The possibility that a B to Z transition of DNA could be important for the interaction of the RNA polymerase with its template is discussed.

The development of gene cloning and in vitro mutagenesis techniques, together with the availability of in vitro transcription systems (Weil et al., 1979; Manley et al., 1989) allowing RNA polymerase B (II) to accurately transcribe cloned DNA templates, have given a new impetus to studies of the control of eucaryotic gene expression. Several DNA sequence elements implicated in the control of initiation of transcription by RNA polymerase B (II) were thus, characterized. An AT-rich region, the Goldberg-Hogness box or TATA box, ordinarily located around 30 nucleotides upstream from the cap site seems to be important in vivo as well as in vitro in limiting initiation to a narrow region (Gluzman et al., 1980; Benoist and Chambron, 1981; Mathis and Chambron, 1981; Grosved et al., 1981). The conserved sequence GATAATCT is often observed around 80 nucleotides upstream from the presumed site for initiation of transcription (Benoist et al., 1980). The cap site itself displays a more or less conserved structure (Busslinger et al., 1980), and it has been reported that RNA polymerase B (II) has a restricted preference for initiation sites harboring a purine flanked by pyrimidine residues (Grosved et al., 1981).

Another relevant observation was the finding of enhancing elements far upstream from the cap site which are needed for in vivo expression but dispensable in the in vitro reconstituted system (for review, Yaniv, 1982).

Although in vitro systems (Weil et al., 1979; Manley et al., 1989) provide a rapid and powerful assay for transcription, they clearly do not observe all of the regulatory properties expected for specific expression. Despite some recent progress (Matsui et al., 1980), the nature and the role of the different cofactors present in the cytoplasmic or whole cell extracts are poorly understood. It remains to be confirmed that all of them are directly related to the control of gene expression and are, in fact, needed in vivo for a specific interaction of the RNA polymerase B (II) with eucaryotic promoters.

In previous studies, it has been reported that purified yeast RNA polymerase B (II) was able to efficiently and selectively initiate in vitro transcription in a dinucleotide primed system (Lescure et al., 1981a, 1981b). We report here the general conditions required for a selective initiation by yeast RNA polymerase B (II) on a recombinant plasmid DNA carrying the yeast alcohol dehydrogenase I structural gene and its surrounding sequences. One of the RNA products synthesized under these conditions has been characterized and its initiation site mapped on the cloned DNA. The homology of the sequence in this region to known yeast genes is surprising.

EXPERIMENTAL PROCEDURES

Enzyme and DNA—Yeast RNA polymerase B (II), Escherichia coli RNA polymerase, and plasmid DNAs were obtained as previously reported (Lescure et al., 1981b). As estimated by gel electrophoresis, the yeast RNA polymerase B (II) used in this work was at least 95% pure. Restriction endonucleases and Klenow DNA polymerase I were purchased from Boehringer and Bethesda Research Laboratories.

In Vivo Synthesis of RNA—Standard reaction mixture (25 μl) for RNA synthesis contained 50 mM Tris-HCl, pH 8, 5 mM (NH4)2SO4, 25 mM MnCl2, 0.5 mM dithiothreitol, and DNA template, RNA polymerase II, and ribonucleosides triphosphate as indicated. After incubation at 30 °C, the reaction was stopped by adding 1 μl of 0.5 M EDTA. For the electrophoretic analysis, the samples were ethanol-precipitated and then resuspended in 5 μl of a buffer containing 80% formamide, 1 mM EDTA, xylene cyanol, and bromphenol blue.

Analysis of RNA Synthesized in Vitro—Electrophoretic analysis was conducted on a 5% polyacrylamide gel containing 7 M urea, 50 mM Tris/borate, pH 8.2, 1 mM EDTA. RNA transcripts fractionated by electrophoresis were eluted from the gel by soaking in 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 0.1% sodium dodecyl sulfate, filtered on a nitrocellulose filter, and electroblotted into nitrocellulose filters. The filters were hybridized with 32P-labeled DNA probes and autoradiographed.

Cloning with M13 mp7—The yeast BamHI DNA fragment of pJ14 (Fig. 1) has been cloned within the BamHI site of M13 mp7 as previously described (Messing et al., 1977, 1981). Hybridization of RNA-labeled probes to gels or blots was performed as described (Thomas et al., 1981).
zation was done in 25 μl of a mixture containing 20 mM Tris-HCl, pH 8, 0.5 M NaCl, 0.5 mM EDTA, and 1 μg of M13 mp7-14 plus or minus single-stranded DNA. After 2 h at 60 °C, 5 μl of the hybridization medium were added to 45 μl of a mixture containing 20 mM Tris-HCl, pH 8, 10 mM MgCl₂, 1 mM dithiothreitol, 0.05 mM (each) dATP, dGTP, dCTP, dTTP, and 0.5 unit of E. coli DNA polymerase I (Klenow). After a 15-min incubation at room temperature, 0.5 unit of BarnHI or AvaII restriction endonucleases was added and incubation continued for 30 min at 37 °C. The reaction was stopped by adding 2 μl of 0.5 M EDTA, and then the samples were ethanol-precipitated and analyzed on a 5% polyacrylamide gel under denaturing conditions.

DNA Sequencing—Labeling of restriction fragments at their 5' ends with [γ-³²P]ATP and subsequent DNA sequence analysis were carried out according to the procedure of Maxam and Gilbert (1977).

RESULTS

Selective Initiation of in Vitro Transcription at Low Nucleotide Triphosphate Concentration—A bacterial plasmid containing the yeast alcohol dehydrogenase gene and surrounding sequences was used as template for in vitro transcription with highly purified yeast RNA polymerase B (II). The physical map of this plasmid pJD14 (Williamson et al., 1980; Lescure et al., 1981b) is described in Fig. 1. When the nucleoside triphosphate concentration was drastically reduced, relative to the standard conditions, the analysis of RNA transcripts on polyacrylamide gels has shown the presence of several discrete low molecular weight bands. A typical result obtained at a nucleoside triphosphate concentration of 5.10⁻⁷ M is shown in Fig. 2A. This result was totally unexpected and suggested that under these particular conditions, purified RNA polymerase B (II) was able to selectively initiate and terminate the in vitro transcription on a supercoiled DNA template. In order to specify the substrate concentrations needed to obtain these results, the nucleoside triphosphates have been tested between 10⁻⁶ and 10⁻⁴ M. The results presented in Fig. 2B show that the pattern of RNA products observed was strongly affected as a function of the substrate concentrations used. Five main products of 60, 62, 101, 142, and 150 nucleotides in length and some minor products have been identified. At the concentration of 10⁻⁶ M, the 60- and 150-mer products were the predominant ones. Progressive increase of the nucleoside triphosphate concentration leads to the synthesis of the 101- and 142-mer products and the decrease of the 60-, 62-, and 150-mer products. This was interpreted as the result of the effect of nucleoside triphosphate concentration on both the initiation of transcription at distinct and limited potential initiation sites and the accumulation of ternary complexes at various pause sites. The kinetics of RNA synthesis at 10⁻⁷ M is shown in Fig. 2C. Under these conditions, there was an accumulation of RNA products, mainly for the 101- and 142-mer RNAs. This suggested that at this substrate concentration, the pause sites corresponded to effective termination sites of the in vitro transcription.

The effect of other experimental conditions affecting the synthesis of the short RNAs has been tested (data not shown). The results can be summarized as follows. 1) At a given NTP concentration, ionic strength and pH do not qualitatively affect the results; the reaction is optimum at pH 8 and concentrations of NaCl higher than 40 mM inhibit the reaction. 2) Whatever the concentration used, Mg²⁺ cannot replace Mn²⁺ as metal ion. 3) Supercoiling of the template is absolutely required.

To gain further insight into the origin of the short RNA products observed, a comparative transcription experiment has been carried out using as template pJD14 DNA or pBR322 DNA, the vector into which yeast alcohol dehydrogenase I DNA had been cloned at the BamHI site (Fig. 1). The transcription was achieved at an intermediate nucleoside triphosphate concentration of 10⁻⁵ M, allowing simultaneous observation of the five main products described above. The results reported in Fig. 2D clearly demonstrated that the 60-, 62-, and 150-mer products originate from pBR322 DNA, whereas the 101- and 142-mer products originate from the yeast DNA.

Hybridization Analysis of pJD14 DNA Transcripts—A more precise analysis of the transcripts obtained from pJD14 DNA at low substrate concentration has been achieved by hybridization to the HindI fragments of pJD14 DNA (Fig. 1). Transcription was carried out for 30 min at a concentration of 10⁻⁵ M NTP. The 60, 62, 101, 142, and 150 nucleotides long ³²P-labeled RNA bands were eluted from a preparative gel and hybridized to the HindI fragments blotted on nitrocellulose. The autoradiogram revealed that the hybridization of 60- and 62-mer products was restricted to fragment G and/or H (Fig. 3). These two fragments of DNA were too close to distinguish them on the blot. The 150-mer product hybridized both to fragment G and/or H and A (Fig. 3). The hybridization of 101-mer product was restricted to fragment B, whereas the 142-mer hybridized to B and D fragment. These results confirmed the location of the initiation sites for 60-, 62-, and 150-mer products within pBR322 DNA and within the yeast DNA fragment for the 101- and 142-mer products (Fig. 2D).

Polarity and Mapping of the 101-mer Product—In order to define the polarity of the short RNA products, the following strategy has been used. The BamHI yeast fragment of pJD14 (Fig. 1) has been cloned within the unique BamHI site of the replicative form of M13 mp7, a derivative of the bacteriophage M13 (Messing et al., 1981). Two recombinant M13 mp7 DNAs, containing the BamHI yeast DNA inserted in opposite orientations, were thus obtained. They were arbitrarily called M13 mp7-14 plus and M13 mp7-14 minus DNAs. The single-stranded DNA of these two M13 recombinants was spotted on a nitrocellulose filter and hybridized with either one of the

**Fig. 1. Diagram of pJD14 DNA with the relevant restriction sites.** The plasmid pJD14 was derived from the original clone pY9T6 of Williamson et al. (1980) by inserting the BamHI DNA fragment containing the gene for yeast alcohol dehydrogenase I into the BamHI site of pBR322 (Lescure et al., 1981b). DNA fragments generated by HindI (A-P) and the location of EcoRI, BamHI, and AvaiI cleavage sites on pJD14 DNA are represented. The arrow represents the location and the polarity of the region coding for the structural gene of yeast alcohol dehydrogenase. The location of the yeast Sau3A DNA fragment of the pJD221 plasmid (Lescure et al., 1981a) is shown. The site for the initiation of the 101-mer product identified in this work is indicated.
In Vitro Transcription by Yeast RNA Polymerase II

Fig. 2. Electrophoretic analysis of RNA products synthesized at low ribonucleoside triphosphate concentrations. In vitro transcription was conducted at 30 °C in the standard reaction mixture containing 1 µg of pJD14 DNA, 0.5 µg of yeast RNA polymerase II, and [α-32P]UTP as label. The 32P-labeled RNA was electrophoresed on a 5% urea-polyacrylamide gel and detected by autoradiography. A, incubations were done for 10 min with 5 × 10−4 M NTP (lanes 1 and 2); 32P HinfI pJD14 DNA fragments have been used as marker (lane 3). B, incubations were done for 10 min at various NTP concentrations. Lane 1, 10−4 M ([α-32P]UTP, 1 µCi/µmol); lane 2, 5.10−5 M ([α-32P]UTP, 0.2 µCi/µmol); lane 3, 10−5 M ([α-32P]UTP, 0.1 µCi/µmol); lane 4, 5.10−6 M ([α-32P]UTP, 0.02 µCi/µmol); lane 5, 10−6 M ([α-32P]UTP, 0.01 µCi/µmol). C, incubations were done at 10−5 M for different periods of time. Lane 1, 2 min; lane 2, 5 min; lane 3, 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 60 min. D, incubations were done for 10 min with 5 × 10−5 M NTP using pJD14 DNA (lane 1), pJD221 DNA (lane 2), or pBR322 DNA (lane 3) as template. In A, the origin of the gel is shown, whereas in B, C, and D is only presented the part of the gel corresponding to a nucleotide length included between 50 and 250.

The autoradiogram of the gel, presented in Fig. 4B, showed that hybridization occurred exclusively with the M13 mp7-14 plus DNA. Therefore, the 101-mer product was transcribed in the opposite direction to that of the alcohol dehydrogenase I structural gene (Fig. 1). This product has been mapped using an approach derived from the primer extension method. The purified 32P-101-mer product was hybridized with the M13 mp7-14 plus DNA. Elongation from this RNA primer was achieved using E. coli Klenow DNA polymerase I fragment and cold deoxyribonucleotide triphosphate as substrates. The extended products were then cleaved by various restriction endonucleases, and the resulting labeled products were analyzed by electrophoresis on a 5% acrylamide gel under denaturing conditions. When the extended products were cleaved by the BamHI restriction endonuclease, a band corresponding to 595 ± 5 nucleotides in length was identified by autoradiography (Fig. 5, lane 1). These results suggested that the 101-mer product was initiated at about 600 base pairs upstream of the BamHI site within the pJD14 Hin/B DNA fragment (Fig. 1). This was further confirmed using the endonuclease AvaII which cleaves the M13 mp7 DNA 326 nucleotides downstream from the BamHI site (Messing et al., 1981). When this enzyme was used to cleave the extended products, the expected band of about 950 nucleotides length was observed (Fig. 5, lane 2).

Nucleotide Sequence of the Initiation Region of the 101-mer Product—The nucleotide sequence (Fig. 6) corresponding
to the region where the 101-mer product was initiated has been determined by the method of Maxam and Gilbert (1977) using the pJD14 HinfI-BamHI DNA fragment 32P 5' end-labeled at the HinfI site (Fig. 1). The presumptive position of initiation of the 101-mer product is deduced from the RNA primer extension experiment (Fig. 5). From previous results (Lescure et al., 1981a) and as discussed, the position zero probably corresponds to the exact location of the 5' end of the 101-mer product.

**DISCUSSION**

The present work contradicts the general assumption that the purified RNA polymerase B (II) initiates transcription at

Fig. 4. Polarity of the 101-mer product. M13 mp7-14 plus and M13 mp7-14 minus DNAs have been obtained as described under “Experimental Procedures.” A, 1 µg of M13 mp7, M13 mp7-14 minus, or M13 mp7-14 plus DNAs was spotted on a nitrocellulose membrane and immobilized by 1-h heating at 80°C. Hybridization with 32P RNA was carried out for 4 h at 60°C in 4 X SSC containing 0.2% sodium dodecyl sulfate. Membrane was washed twice in 4 X SSC containing 0.2% sodium dodecyl sulfate and then submitted to autoradiography. Lane 1, 32P RNA obtained after 2 h of *in vitro* transcription at 37°C of pJD14 DNA by *E. coli* RNA polymerase; lane 2, 32P-101-mer product eluted from polyacrylamide gel. B, the pJD14 HinfI DNA fragment (Fig. 1) was 5' end-labeled using [γ-32P]ATP and 5' polyacrylamide kinase. Secondary cleavage was achieved by BamHI endonuclease (Fig. 1) and the larger fragment (B') was purified by electrophoresis on a 5% polyacrylamide gel. Heat-denatured B' fragment was then hybridized in 25 µl of 4 X SSC to 1 µg of M13 mp7-14 minus (lane 1) or M13 mp7-14 plus DNAs (lane 2) for 2 h at 60°C. The formation of hybrids was analyzed by electrophoresis on a 1% agarose gel and autoradiography.

Fig. 5. 5' end mapping of the 101-mer product. The strategy for determining the 5' end of the 101-mer product is summarized in A. The conditions under which the experiments were conducted are described in detail under “Experimental Procedures.” Results are presented in B. Cleavage of extended products by BamHI (lane 1) or AvaI (lane 2) endonucleases. Lane 3, control experiment without extension and cleavage of 32P RNA primer. Lane 4, pJD14 HinfI 32P DNA fragments used as size marker.
Fig. 6. Nucleotide sequence of the initiation region of the 101-mer product. The nucleotide sequence is $5'$ to $3'$ clockwise from the HindIII site between the HindIII and HindII fragments (Fig. 1). The position 0 corresponds to the presumed initiation site. The $5'$ end of the 101-mer product assigned by the RNA primer extension mapping (Fig. 5) is shown by $\cdot$. The arrow indicates the probable location of the 11-mer product obtained when the in vitro transcription on pJD14 DNA was primed by the dinucleotide UApA and elongated in the absence of GTP (Lescure et al., 1981a). The large blocks of alternated purine and pyrimidine residues are underlined. The Sau3A site showing the limit of the yeast DNA insert in the plasmid pJD221 (Lescure et al., 1981a) is indicated.

random sites on DNA template (Chambon, 1974; Roeder, 1975). We have shown that under appropriate conditions, the in vitro transcription by pure yeast RNA polymerase B (II) was restricted to a few sites only on a defined template.

General Conditions for Selective Initiation—The present in vitro system has particular features which are absolute requirements in order to observe a selective transcription in vitro. First, the concentration of nucleoside triphosphate has to be kept low. Second, the template has to be in a supercoiled state. Third, Mn$^{2+}$ is used as divalent cation. The problem is to understand how these parameters affect the selectivity of the initiation.

Previous studies (Lescure et al., 1981b) have shown that the pJD14 DNA was able to selectively direct a very efficient synthesis of the trinucleotide UpApU using UpA as primer and UTP as substrate. The apparent $K_m$ for UTP in this reaction was about $0.5 \times 10^{-5}$ M. Moreover, we have shown that yeast RNA polymerase B (II) was able to initiate transcription at a concentration of NTP lower than $10^{-5}$ M (Fig. 2B). These concentrations are about 2 orders of magnitude lower than those ordinarily used for in vitro transcription in reconstituted systems (Weil et al., 1979; Manley et al., 1980). Increasing NTP concentrations causes a progressive disappearance of the small abortive products and an increase of the background (Fig. 2B). Therefore, the substrate concentration is probably important for two reasons. The first one is to restrict the initiation at low nucleoside triphosphate concentration to only a few strong sites, higher concentrations leading to a random initiation on the template. The second one is to permit RNA polymerase to pause at defined sites, allowing the formation of discrete products which can be analyzed. It must be noted that the substrate concentration affected differently the various initiation and pause sites identified (Fig. 2B). Therefore, the ribotriphosphate concentration must be adjusted to an optimum for the optimal formation of a given product.

The second feature required to obtain the selective transcription was the supercoiled state of the template. Negative supercoiled DNA is torsionally stressed. One way to minimize its free energy is to denature an AT-rich section. Since RNA polymerase B (II) preferentially initiates transcription on single-stranded DNA (Dezéée et al., 1974), it has been sug-

1 B. Lescure, P. Cotrelle, and A. Sentenac, manuscript in preparation.

2 B. Lescure, unpublished results.
handed conformation of DNA has been identified in the interband regions of Drosophila polytene chromosomes (Nordheim et al., 1981).

The assumption that a right-handed to left-handed DNA transition could be important for the interaction of the RNA polymerase II (II) with the template is supported by the results presented in Fig. 7 showing that there is a strong stimulation of the enzyme activity on poly(d(A-T)) in MgCl₂/ethanol. It has been recently reported that the interconversion between the right- and left-handed helical forms of poly(d(G-C)) occurs at low concentrations of MgCl₂ and ethanol, acting together in a highly synergistic manner (Van de Sande and Jovin, 1982).

**Structure of DNA Template at the 101-mer Initiation Site—**The RNA primer extension methodology that we have developed (Fig. 5) is particularly suitable for the mapping of the products synthesized in our system. This method has allowed us to map the initiation site of the 101-mer product at 595 ± 5 nucleotides from the BamHI site within pJD14 HinfIB DNA fragment (Figs. 1 and 6). Alignment of this site on the sequence which has been determined for this region (Fig. 6) is in good agreement with previous results. 1) pJD14 DNA directed a very efficient UpAgU synthesis in the presence of Upₐ and UTP. This synthesis was 10 to 20-fold decreased when pBR322 or pJD221 plasmid DNAs, in which this region was deleted (Figs. 1 and 6), were used as templates (Lesure et al., 1981b). 2) Upₐ-primed oligonucleotide synthesis on pJD14 DNA in the absence of GTP led to the formation of four main products of 11, 14, 17, and 20 nucleotides in length, respectively, whereas under similar conditions, the 11-mer product was absent when pJD 221 was used as template (Lesure et al., 1981a). The presumed initiation site is followed by 11 residues before the first G residue in the sequence.

The examination of the DNA sequence in the initiation region for the 101-mer product reveals several interesting features which are summarized in Fig. 8.

The sequence TATATATAT is found 40 nucleotides upstream from the initiation site for the 101-mer product. The sequence CACAC which is present at a similar position in several other yeast RNAs (Holland and Holland, 1979; Smith et al., 1979, glycerolaldehyde-3-phosphate dehydrogenase (Holland and Holland, 1979), histone H2B2 (Wallis et al., 1980), iso-2-cytochrome c (Montgomery et al., 1980), and tryptophane I (Tchumper and Carbon, 1980), but are absent for others like actine, mating type a or α genes. For the iso-1-cytochrome c gene, the deletion of the major part of these blocks at 15 nucleotides upstream from the TATA box reduced 15-fold the in vitro expression of this gene (Faye et al., 1981).

Two potential initiation codons AUG are found, respectively, 18 and 37 nucleotides downstream from the initiation site for the 101-mer product. The second one is preceded by the sequence CACAC which is present at a similar position in several other yeast RNAs (Holland and Holland, 1979; Smith et al., 1979, Tchumper and Carbon, 1980; Stiles et al., 1981). Furthermore, the AUG codon is found in the sequence 5'-CAAUGACCUUU'-7, which is complementary to the conserved 3' terminus of 18 S rRNA from eucaryotic cells, 3' HGUUACUAGGAAG 5' (Hagenbuche et al., 1978). Moreover, the 200 residues preceding this ATG contain nine translation termination codons distributed in each of the three possible reading frames (Fig. 6). The sequence CTATAC found at the initiation site for the 101-mer product is also observed for the major cap sites of the iso-1-cytochrome c gene (Faye et al., 1981) and of the alcohol dehydrogenase I gene (Bennetzen and Hall, 1981).

These observations are in favor of a biological significance of the selective initiation site that we have identified in this work. However, this assumption remains to be confirmed by experiments in vivo. From the polarity of transcription, it was clear that the 101-mer mRNA was not related to the transcription of the alcohol dehydrogenase I gene. It has been reported that the 5' end of the alcohol dehydrogenase I mRNA mapped at -27 and -37 nucleotides upstream from the AUG initiation codon (Bennetzen and Hall, 1981). However, according to the growth conditions, a remote initiation site is located at least 1200 base pairs upstream from the AUG codon. Preliminary results (data not shown) suggest that another strong in vitro initiation site on pJD14 DNA is located in the HinfIB DNA

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1. B. Hall and G. Amerer, personal communication.
fragment (Fig. 1) in the opposite polarity to that of the 101-mer RNA. This site could be related to the expression of the ADH I gene.

Studies are in progress to determine the location of the other selective initiation sites described in this work. The analysis of the corresponding DNA structures could contribute to understanding the nature of the RNA polymerase B (II) with its template. The examination of the biological significance of the sites we observed will be undertaken. If this was the case, the system that we have described in this work would contribute to a considerable simplification of the reconstituted systems for in vitro transcription and, therefore, permit more precise studies of the molecular events implicated in the regulation of genetic expression.

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