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ABSTRACT In both *Escherichia coli* and *Salmonella typhimurium* there is approximately balanced synthesis of the six catalytic and six regulatory polypeptide chains of the regulatory enzyme aspartate transcarbamoylase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2). This control is achieved by the contiguous *pyrB* and *pyrI* genes, which encode the catalytic and regulatory chains, respectively. Evidence for this single transcriptional unit was obtained by a study of various deletion mutations and from the DNA sequence of the intercistronic region between *pyrB* and *pyrI*. One *pyrB* deletion, *pyrB748*, produced a normal level of regulatory chains even though it removed a substantial portion of the *pyrB* gene. Another deletion, *pyrB740*, shares a similar terminus at one end within *pyrB*, but the promoter region is removed. In this deletion mutation, there is no production of the regulatory polypeptide, indicating that a single region adjacent to *pyrB* controls transcription of *pyrI* as well. Molecular cloning and subsequent DNA analysis demonstrated that the *pyrB* and *pyrI* genes are contiguous with *pyrI* as the distal gene in the operon. The cistrons are separated by a 15-nucleotide untranslated region containing a sequence capable of interacting with the 16S ribosomal RNA and allowing translation of the *pyrI* cistron.

It has been known for many years that the allosteric enzyme aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase; carbamoylphosphate:L-aspartate carbamoyltransferase; EC 2.1.3.2) from *Escherichia coli* is an oligomer composed of six catalytic (c) and six regulatory (r) polypeptide chains (1–6). Moreover, there is approximately balanced biosynthesis of the chains in both *E. coli* (7) and *Salmonella typhimurium* (8). These observations led to the suggestion that the structural genes for the c and r chains (*pyrB* and *pyrI*, respectively) are organized as an operon (8) and that biosynthesis involves a polycistronic messenger RNA (7). However, precise knowledge of the location of the *pyrI* gene and definitive evidence that both genes are subject to the same transcriptional control have not been available.

ATCase of both *E. coli* and *S. typhimurium* is composed of two trimeric catalytic (C) subunits and three dimeric regulatory (R) subunits (1–6, 9, 10). Isolated C trimers of molecular weight 100,000 are catalytically active but insensitive to the feedback inhibitor, CTP, and the activator, ATP (1), whereas the free R dimers of molecular weight 34,000 are devoid of catalytic activity but still bind both CTP and ATP with high affinity (1). When C and R subunits are mixed, reconstituted ATCase of molecular weight 300,000 is formed in high yield, and the complex exhibits the allosteric properties of native ATCase (1).

Several lines of evidence indicate that the *pyrI* gene is in close proximity to the *pyrB* gene. Single deletions in *S. typhimurium* eliminate production of both c and r polypeptide chains (8). In addition, specialized λ phages capable of transducing the *pyrB* gene also encode the r chain (11), but the distance between *pyrB* and *pyrI* is uncertain. Although the genes for the biosynthetic pathway (*pyrB*, *pyrC*, *pyrD*, *pyrE*, and *pyrF*) respond in a coordinate manner to uracil starvation (12), they map at different positions (13). The increase in ATCase activity, reflecting the elevated and balanced expression of *pyrB* and *pyrI*, is at least 10-fold greater than that of the other enzymes (12, 14).

Support for the suggestion that *pyrB* and *pyrI* are part of the same transcription unit stems from two independent sets of experiments. Perbal and Hervé (7) determined the kinetics of incorporation of radioactively labeled amino acids into the c and r chains during and immediately after uracil starvation, and on the basis of the results they proposed that synthesis of both types of polypeptide chains was directed by a polycistronic messenger RNA in *E. coli*. Further evidence for an operon structure was provided by Syvanen and Roth (8) in their demonstration of the polarity of *pyrB* chain-terminating mutations on the synthesis of r chains in *S. typhimurium*.

In order to eliminate uncertainty regarding the organization of the *pyrB* and *pyrI* genes and the control of their expression, we have analyzed the effect of *pyrB* deletion mutations on the synthesis of the r polypeptide chains. These studies have shown that *pyrI* is subject to the same transcriptional regulation as *pyrB* even in the absence of the *pyrB* product. In addition, we have determined the position of the *pyrI* gene by DNA sequence analysis of the intercistronic region between *pyrB* and *pyrI*. These results establish that the contiguous *pyrB* and *pyrI* genes constitute a single transcriptional unit encoding the c and r chains of *E. coli* ATCase.

MATERIALS AND METHODS

Bacterial Strains and Their Construction. All *S. typhimurium* strains listed in Table 1 are derivatives of LT2 except TR3200, which was derived from LT7. The *pyrH700* mutation results in a partially defective UMP kinase; as a consequence the levels of UDP and UTP are reduced, resulting in greatly increased production of ATCase (9, 15–17). Mutation *pyrB655* is a deletion that removes all of *pyrB* (8). The F393 episome is derived from F128 (F′ *pro lac*) of *E. coli* K-12 and contains the *E. coli pyrB* (17). Media, transductional methods, and conjugational transfers were described earlier (17). High-titer phage P22 lysates for mapping experiments were concentrated 10-fold to yield titers of about 10^{11} plaque-forming units/ml; they were stored in T-2 buffer (18).

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Abbreviations: ATCase, aspartate transcarbamoylase; c, catalytic polypeptide chain; r, regulatory polypeptide chain; C, catalytic subunit; R, regulatory subunit; Pyr$^+$, pyrimidine-independent; kb, kilobase.

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Table 1 Bacterial strains

Strain	Genotype
S. typhimurium	
HS2230	*argI2002 fol-101 leuD798 proAB47 pyrB655 pyrH700*
HS2231	*argI2002 fol-101 leuD798 proAB47 pyrB655 pyrH700/* F393 *lac⁺ pro⁺ argF280* (P22 *pyrB⁺*)
HS2255	*argI2002 fol-101 leuD798 proAB47 pyrB655 pyrH700/* F393 *lac⁺ pro⁺ argF282::Tn10* (P22 *pyrB740*)
HS2256	*argI2002 fol-101 leuD798 proAB47 pyrB655 pyrH700/* F393 *lac⁺ pro⁺ argF282::Tn10* (P22 *pyrB⁺*)
HS2273	*argI2002 fol-101 leuD798 proAB47 pyrB655 pyrH700/* F393 *lac⁺ pro⁺ argF282::Tn10* (P22 *pyrB748*)
HS2278	*argI2002 fol-101 leuD798 proAB47 pyrB655 pyrH700/* F393 *lac⁺ pro⁺* (P22 *pyrB754*)
HS2306	*fol-101 leuD798 proAB47 pyrB655 pyrH700/* F393 *lac⁺ pro⁺ argF282::Tn10* (P22 *pyrB748*)
HS2307	*fol-101 leuD798 proAB47 pyrB655/* F393 *lac⁺ pro⁺ argF282::Tn10* (P22 *pyrB748*)
TR3200*	*amtA1 proAB47 pyrB655 trp-130*
E. coli	
AT2535†	*pyrB59, argH2, thi-1, his-1, purF1, mtl-2, xyl-7, malA1, ara-13, lacY1* or *lacZ9, rps-18, 9* or *14, tonA2* or *ton-14, tsx-23* or *tsx-25, λ^r, λ^-, supE44*

* Kindly provided by J. R. Roth.
† Obtained from the *E. coli* Genetic Stock Center (New Haven, CT).

Isolation and Mapping of Deletion Mutants. Deletion mutants HS2255 (*pyrB740*), HS2273 (*pyrB748*), and HS2278 (*pyrB754*) are spontaneous derivatives of HS2256 obtained by selecting for *pyrB* mutants as suppressors of arginine auxotrophy (17). These deletion mutants are completely auxotropic for pyrimidines. Mapping was performed by transductional crosses using high-titer P22 stocks grown on point mutants and selecting for the ability of the deletion mutants to form pyrimidine-independent (Pyr^+) transductants in spot tests on selective media. Endpoints were confirmed by crosses using 100–200 μl of high-titer P22 stocks and 100 μl of recipient cells that had been concentrated 10-fold after overnight growth in nutrient broth supplemented with a 2% vol of E medium (at 50 times the normal concentration). The crosses were scored after 48 hr at 37°C. In crosses in which recombination was possible at least 100 colonies resulted, whereas negative results were indicated by a complete absence of Pyr^+ transductants.

Preparation of Cell Extracts. Bacteria for assays of R subunit were grown to late logarithmic phase in 25-ml cultures of E medium supplemented with 0.25% glucose, uracil at 20 μg/ml, and required amino acids at 100 μg/ml. Extracts were prepared as described by Syvanen and Roth (8). Protein concentrations in the extracts were 0.8–1.5 mg/ml.

Preparation of Radioactively Labeled C Subunit. ^{125}I-Labeled C subunit was prepared by the method of Syvanen *et al.* (19), using 0.5 mCi (1 Ci = 3.7×10^{10} becquerels) of $Na^{125}I$ (Amersham) and 40 μg of purified C subunit from *E. coli* ATCase. The specific activity of the labeled protein was 4.2×10^6 cpm/μg.

Assay for R Subunit in Cell Extracts. The amount of R subunit in the extracts was measured by the procedure (assay II) found to be present in the extracts in preliminary experiments. This procedure was followed in order to obtain the maximal sensitivity consistent with a broad range of detection. The reconstitution of ATCase was achieved by incubating the mixtures of subunits for 30 min at 30°C. In all experiments the labeled C subunit was in excess of any unlabeled C subunit in the extracts, and not more than 50% of the ^{125}I-labeled subunit was converted to radioactively labeled ATCase. Unlabeled ATCase and C subunit were added as markers and the samples were subjected to electrophoresis in 5-cm polyacrylamide gels (5%). After electrophoresis the gels were stained and sliced, and the distribution of radioactivity in ATCase and C subunit was determined.

Plasmid Construction. Restriction endonucleases were obtained from New England BioLabs and used according to the supplier's specifications. The bacterial strain AD11m5, carrying the λ specialized transducing phage yk14m5 (λd *pyrB argI valS*) as a prophage (20) was kindly provided by Aki Kikuchi. Bacteriophage DNA was isolated by the procedure of Wu *et al.* (21). Plasmid DNA was isolated by the alkaline-$NaDodSO_4$ extraction method of Birnboim and Doly (22), and the DNA was purified further by centrifugation in a CsCl/ethidium bromide gradient (23).

A molecular recombinant plasmid containing the *pyrB* gene was constructed by digestion of yk14m5 and pBR322 (24) DNA with the restriction endonucleases *Pst* I and *Eco*RI and subsequent ligation of the fragments with phage T4 DNA ligase. Plasmids were used to transform strain AT2535 (carrying the *pyrB59* allele) made competent by treatment with $CaCl_2$ (25). The resulting transformants were selected as Pyr^+ tetracycline-resistant colonies. One isolate was selected and the 7.4-kilobase (kb) plasmid, pDP7, was purified and analyzed by restriction endonuclease digestion and electrophoresis in 0.7% agarose gels.

The size of the DNA fragment containing the *pyrB* gene was reduced by digesting plasmid pDP7 with *Eco*RI and treating the linear DNA with *Bal* 31 double-stranded exonuclease (26) for 30 min at 30°C. Incubation of the resulting DNA overnight with T4 DNA ligase yielded plasmid pDP8, which was used to transform AT2535 cells, selecting for pyrimidine prototrophy. Isolates were screened to ensure that they were tetracycline sensitive because exonucleolytic digestion of pDP7 from the *Eco*RI site should result in the loss of tetracycline resistance (27). The ability of plasmid pDP8 to encode both c and r polypeptides was tested as follows: A derivative of TR3200 (carrying the *pyrB655* deletion) containing pDP8 was constructed. Cellular extracts were prepared and subjected to electrophoresis in 5% polyacrylamide gels. The mobilities of the enzymically active proteins were determined by using the activity stain described by Bothwell (28). As little as 1 ng of active protein (ATCase or C subunit) can be detected readily by this procedure.

Sequence Determination. A DNA fragment approximately 650 base pairs in length was derived by digestion of pDP8 with the restriction endonuclease *Msp* 1 (Fig. 1) and was labeled at the 5′ termini with [γ-^{32}P]ATP (Amersham, >5,000 Ci/mmol) and polynucleotide kinase (P-L Biochemicals). The DNA strands were separated according to the procedure of Maxam and Gilbert (29). In other experiments the plasmid was digested

yk14m5) by linearization of the molecule by *Eco*RI digestion, treatment with *Bal* 31 double-stranded exonuclease, and ligation with T4 DNA ligase. Only the segment containing *pyrB* and *pyrI* is shown. The junction with pBR322 after *Bal* 31 digestion is indicated by Junct, and the restriction sites utilized to produce fragments for nucleotide sequence determinations are shown. Arrows represent the sites cleaved by *Msp* I to yield the fragment containing 650 base pairs. The fragment produced by *Bgl* II/*Pvu* II contained 950 base pairs.

determined by the method of Maxam and Gilbert (29) with the modifications described by Smith and Calvo (30).

RESULTS

Mapping of Deletion Mutations. Twenty-four *pyrB* deletion mutants have been isolated as part of the construction of a detailed recombination map of the *pyrB* locus; only three are described here. These deletions, *pyrB740*, *pyrB748*, and *pyrB754*, were mapped by transductional crosses with point mutations whose map positions had been determined independently (31) and are shown in Fig. 2. In this map, *pyrB554* is the most promoter-proximal point mutation that has been mapped (31). Deletions *pyrB740* and *pyrB748* fail to recombine with point mutations from *pyrB554* through *pyrB717*. However these two deletions recombine with all point mutations from *pyrB727* through the end of *pyrB*. A *pyrB754* strain is able to form Pyr^+ transductants when donors carry point mutations from *pyrB554* through *pyrB731*, but *pyrB754* does not recombine with *pyrB713* or *pyrB730*. These results are summarized by the map shown in Fig. 2.

The deletion *pyrB754* extends at least to *argF* on the F393 episome because it has simultaneously become Pyr^- and tetracycline sensitive (the transposon Tn*10* in *argF* of the parent strain confers tetracycline resistance). Because of the deletion, *argF* has become 100% linked with *pyrB* by P22-mediated transduction (342 of 342 Pyr^+ transductants had become arginine independent), as contrasted with the original cotransduction frequency of 7% (for *pyrB554*, only 24 of 342 Pyr^+ transductants were arginine independent). These results confirm the extent of the deletion *pyrB754*.

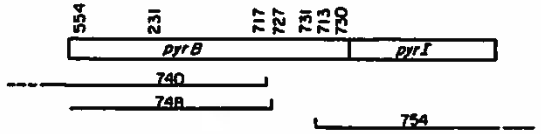

FIG. 2. Map of *pyrB* deletions. Relevant point mutations from the fine structure recombination map of the *pyrB* gene obtained by three-factor crosses (31) are presented at the top. Shown below are the three partial deletions of *pyrB* utilized in this work; endpoints are defined by the nearest point mutations. Both *pyrB740* and *pyrB748* have their right endpoints between *pyrB717* and *pyrB727*. Because *pyrB727* recombines with *pyrB748* at a lower frequency than with *pyrB740*, the end of deletion *pyrB748* is probably closer to *pyrB727* than is the endpoint of *pyrB740*. Deletions *pyrB740* and *pyrB748* fail to recombine with *pyrB554*, the most promoter-proximal point mutation that has been mapped. Deletion *pyrB740* continues leftward through the promoter for *pyrB*, whereas deletion *pyrB748* does not (see text). Point mutation *pyrB231* has been shown to correspond to amino acid residue 125 in the c polypeptide chain (32). Mutation *pyrB754* deletes the carboxyl-terminal coding region of *pyrB*, all of *pyrI*, and the flanking region at least to *argF*. Transcription is from left to right.

HS2278	*argI2002 pyrB655 pyrH700/F' pyrB754*	<0.2
HS2306	*pyrB655 pyrH700/F' pyrB748*	7.5
HS2307	*pyrB655/F' pyrB748*	<0.2

* Amounts of R subunit per mg of cell protein in the cell extracts were determined by the procedure of Syvanen and Roth (8). Amounts less than 0.2 μg/mg were below the level of detection.

Production of R Subunit in *pyrB* Deletion Strains. The different strains containing deletions in *pyrB* were assayed for the production of R subunit (as described in *Materials and Methods*) in order to determine which regions were necessary for *pyrI* expression. Table 2 summarizes the results of these assays in terms of the amount of R subunit in crude extracts.

HS2230, carrying only the deletion *pyrB655*, produced no detectable R subunit, as expected from the results of Syvanen and Roth (8). In contrast, HS2231, with a normal *pyrB* gene on the episome, produced a large amount of R subunit because of the effect of the *pyrH700* mutation (17). Deletions *pyrB740* and *pyrB754* eliminated the production of R subunit.‡ However, a large quantity of R subunit was produced in HS2273, which carries the deletion *pyrB748*. This mutation does not interfere with the normal control of R subunit synthesis, as shown by the results with HS2307. In this $pyrH^+$ derivative virtually no R subunit is detectable. The effect of *pyrH700* on the production of R subunit in the absence of the *pyrB* product is consistent with the increase observed in ATCase production in $pyrB^+$ strains caused by *pyrH700* (17). The overproduction of R subunit in HS2273 is due primarily to the *pyrH700* allele rather than *argI2002*, as shown by the comparison of the amounts of R subunit in the extracts of HS2306 and HS2307.

Structure of pDP8. The plasmid pDP8 was mapped by digestion with the *Pst* I, *Bgl* II, and *Pvu* II restriction endonucleases. The extent of the *Bal* 31-induced deletion was estimated by digestion with *Hin*fI and *Taq* I, placing the endpoint between nucleotides 851 and 1,005 of pBR322 (33). Nucleotide sequence determinations (34) show the junction to be at nucleotide 874 of the pBR322 sequence (27). The total size of the deletion is 2 kb, indicating that pDP8 is 5.4 kb. Polyacrylamide gel electrophoresis of cellular extracts from a TR3200 derivative containing pDP8 revealed a single band when the gels were stained for enzyme activity (ref. 28; see *Materials and Methods*). The protein responsible for the activity had the electrophoretic mobility of native ATCase rather than free C subunit. Thus the ability of pDP8 to direct the synthesis of intact ATCase in the TR3200 background was the result of the presence of both *pyrB* and *pyrI* genes in the plasmid.

Sequence of the Intercistronic Region. The nucleotide sequence of parts of the *pyrB* and *pyrI* structural genes and the intercistronic region between them is shown in Fig. 3. Beginning with the initiation trinucleotide, ATG, the predicted se-

‡ Similar results were obtained for nine additional deletions. Five deletions extend in the same direction as *pyrB740* with right endpoints at five widely separated locations in *pyrB*. The other four delete different amounts of the promoter-distal end of *pyrB*.

Ala-Leu-Val-Leu-Asn-Arg-Asp-Leu-Val-Leu-Stop Met-Thr-His-Asp-Asn-Lys-Leu-Gln-Val

GCA-CTG-GTT-CTG-AAT-CGC-GAT-CTG-GTA-CTG-TAAGGGGAAATAGAGATG-ACA-CAC-GAT-AAT-AAA-TTG-CAG-GTT

FIG. 3. Partial nucleotide sequence of the 650-base DNA fragment containing parts of *pyrB* and *pyrI* and the intercistronic region. The sequence reads from the 5′ direction on the left to the 3′ direction on the right. Amino acids shown above the DNA sequence represent the carboxyl-terminal region of the c chain and the amino-terminal region of the r chain.

quence of amino acids (shown above the DNA sequence in the 5′-to-3′ direction) is identical to the amino terminus of the r polypeptide chain determined by Weber (2). The nucleotide sequence preceding the termination codon, TAA, is thought to constitute the coding region for the carboxyl terminus of the c polypeptide chains for the following reasons: First, the terminal amino acid corresponding to the trinucleotide CTG is leucine, which has been identified as the carboxyl-terminal amino acid in the c chains (2). Second, the 650-base-pair *Msp* I fragment contains a nucleotide region encoding the peptide Arg-Leu-Asp-Pro-Ser-Glu-Tyr-Ala-Asn-Val-Lys, described by Landfear *et al.* (35) as the amino acid residues from positions 207 to 217 in the c chains. This nucleotide sequence is in the same reading frame 192 base pairs from the carboxyl terminus in Fig. 3. Third, a DNA fragment adjacent to the *Msp* I fragment described here has a nucleotide sequence (34) that would encode a peptide identical to the 38 amino acid polypeptide whose sequence was determined by Wall and Schachman (32).

Although the complete nucleotide sequence of the *pyrB* gene and the amino acid sequence of the c chains are not as yet available, the evidence cited here is sufficient to warrant the conclusion that the nucleotide sequence in Fig. 3 includes regions of the *pyrB* and *pyrI* structural genes as well as the intercistronic region.

DISCUSSION

Studies aimed at demonstrating an operon encoding the c and r chains of ATCase (7, 8) have been hindered by the lack of direct information concerning the linkage of the *pyrB* and *pyrI* genes and the inability to isolate regulatory mutants of the *cis*-dominant type. From a study of various deletion mutations we have established that a region genetically linked to *pyrB* regulates the production of both c and r polypeptide chains in a single transcriptional unit. Moreover, the structural organization of the *pyrB* and *pyrI* genes was determined by analysis of the DNA sequence of the intercistronic region.

The *pyrB748* mutation, as seen in Fig. 2, removes a large portion of the *pyrB* gene. Nonetheless there is normal synthesis of the r polypeptide. In a *pyrB748* strain carrying the unlinked *pyrH700* mutation there is a large production of R subunit (Table 2) despite the lack of synthesis of C subunit. In contrast, the synthesis of R subunit is very low in a *pyrB748* strain lacking the *pyrH700* mutation (strain HS2307). These observations on the production of R subunit are very similar to those for R production in comparable backgrounds of $pyrB^+$ strains, in which the R subunit exists as ATCase (17). We conclude from these results that the normal function of the regulatory region for *pyrI* is unaffected by *pyrB748* and that possible *trans* effects of the c polypeptide on *pyrI* expression are highly unlikely.

As shown in Table 2, both *pyrB754* and *pyrB740* mutations eliminate production of the R subunit. In the case of *pyrB754* the absence of the r polypeptide is attributed to the deletion

and *pyrI* are contiguous genes. These results account for the polarity data of Syvanen and Roth (8) for *S. typhimurium* and the observations with the various deletions discussed above. Additional sequence information (34) confirms the gene order shown in Fig. 1 with the *pyrB* cistron closer to the *Pst* I endonuclease recognition site and transcription away from that site. Because *pyrB* was isolated on a *Pst* I/*Eco*RI fragment from the transducing phage yk14m5 (see *Materials and Methods*) and a 16.4-kb *Eco*RI fragment of this phage contains both the *pyrB* and *argI* genes (34), the *Pst* I end of the cloned fragment must be closer to *argI*. Therefore, as reported for *S. typhimurium* (8), transcription proceeds away from *argI*, in the so-called counterclockwise direction on the *E. coli* chromosome (36).

The 15-nucleotide untranslated region separating *pyrB* and *pyrI* differs from those for *trpE–trpD* (37) and *trpB–trpA* (38), whose products are needed in stoichiometric amounts to form anthranilate synthase and tryptophan synthase, respectively. For both of these pairs of genes the intercistronic region contains only two untranslated nucleotides. Oppenheim and Yanofsky (39) suggested that the small intercistronic regions would allow "translational coupling" of the genes by a mechanism in which one of the ribosomal subunits remains associated with the mRNA throughout translation of both structural genes, thereby leading to stoichiometric production of the polypeptides. In the case of the *pyrB–pyrI* operon the intercistronic region

*T-*A-*A-*C-*G-C-*G-A-A-A-T-A-G-A-G

contains six nucleotides (indicated by asterisks) that have a sequence complementary to that of the 16S ribosomal RNA considered to be important in the initiation of translation. This result suggests that translational coupling probably does not occur in the *pyrB–pyrI* operon by the same mechanism as in the *trp* operon, even though the products of these genes are all needed in stoichiometric amounts to form the oligomeric complexes.

Our findings demonstrate that the *pyrB* and *pyrI* genes are contiguous and are transcribed under the control of the same promoter. They are separated by a short intercistronic region containing the translational terminator for *pyrB* and a potential ribosome binding site for *pyrI*. These results unambiguously establish that *pyrB* and *pyrI* make up an operon encoding the catalytic and regulatory chains of ATCase. Completion of the nucleotide sequence for this operon should yield additional information on the structure of ATCase and the regulation of its synthesis.

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