CHAIN INITIATION IN A POLYCISTRONIC MESSAGE: SEQUENTIAL VERSUS SIMULTANEOUS DEREPRESSION OF THE ENZYMES FOR HISTIDINE BIOSYNTHESIS IN SALMONELLA TYPHIMURIUM

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The pathway for histidine biosynthesis in *Salmonella typhimurium*, elucidated largely through the efforts of Dr. B. N. Ames and co-workers,[1-5] is shown in Figure 1. The figure also shows that portion of the pathway for purine biosynthesis which involves the further metabolism of 4-amino-5-imidazole carboxamide ribonucleotide (phosphoribosyl-AIC).[6] Dr. Phillip Hartman and his colleagues have shown that the structural genes for the enzymes catalyzing the reactions of the histidine pathway are localized in a small segment of the bacterial chromosome, the *histidine operon*.[7, 8]

The phenomenon of coordinate derepression of the histidine enzymes was originally described by Ames and Garry,[9] who found that the histidine operon functions as a single unit in response to the level of histidine available to the organism. Evidence presented by Martin[10] indicates that the histidine operon is transcribed into a polycistronic messenger-RNA, and studies on polarity in the histidine operon[7, 11, 12] suggest that this messenger-RNA is translated from the end which

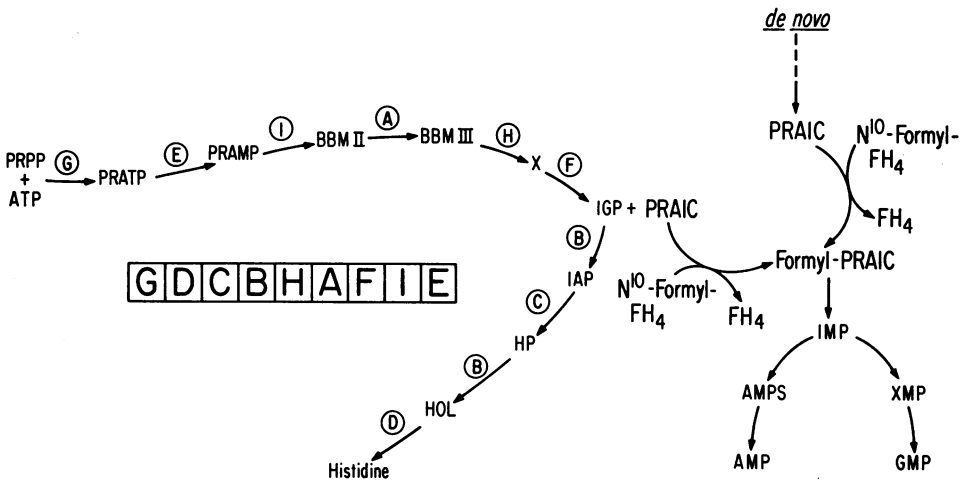

FIG. 1.—Schematic representation of the relationship between the pathways for histidine and purine biosynthesis in *Salmonella typhimurium*, with the order of genes in the histidine operon shown in the center. The letters indicate the genes of the histidine operon which specify the enzymes catalyzing these reactions. The abbreviations used are: PRPP, 5-phosphoribosyl-1-pyrophosphate; PRATP and PRAMP, N-1-(5′-phosphoribosyl)adenosine tri- and monophosphate; BBM II, N-(5′-phospho-D-ribosylformimino)-5-amino-1-(5″-phosphoribosyl)-4-imidazole carboxamide; BBM III, N-(5′-phospho-D-1′-ribulosylformimino)-5-amino-1(5″-phosphoribosyl)-4-imidazolecarboxamide; IGP, D-erythro-imidazolglycerol phosphate; IAP, imidazole acetol phosphate; HP, L-histidinol phosphate; HOL, L-histidinol; N^{10}-Formyl-FH_4, N^{10}-formyltetrahydrofolate; Formyl-PRAIC, formylphosphoribosyl aminoimidazole carboxamide; IMP, inosinic acid; AMPS, adenylosuccinic acid; AMP, adenylic acid; XMP, xanthylic acid; GMP, guanylic acid.

has recently been identified as the operator end.[13] Thus, it appeared that utilization of the information encoded in the histidine operon is oriented in a specific direction. In order to determine whether this orientation could be reflected in a temporal sequence of events, the kinetics of derepression of the histidine enzymes were investigated in a wide variety of histidine auxotrophs.[14, 15] These studies revealed that derepression may proceed by two modes, depending upon which enzyme in the pathway for histidine biosynthesis is affected by the mutation. Derepression of the enzymes occurred simultaneously in those mutants incapable of producing phosphoribosyl-AIC as a by-product of the histidine pathway. Derepression of the enzymes occurred in a temporal sequence corresponding to the positional sequence of the genes in the histidine operon in those mutants which do produce phosphoribosyl-AIC via the histidine pathway.[15]

On the basis of our studies on the kinetics of the derepression process, we advanced the hypothesis that under conditions in which the *sequential* mode of derepression is observed, the polycistronic message transcribed from the histidine operon is translated only from the operator end, whereas under conditions in which the *simultaneous* mode of derepression is observed, the translation of this message is initiated at multiple sites.[15] We now present evidence in support of this hypothesis which indicates that the mode of derepression is determined by the availability of formyl groups for polypeptide chain initiation.

Materials and Methods.—The mutants employed in the present study were auxotrophs derived from *Salmonella typhimurium*, strain LT-2. The wild-type strain (LT-2) and the histidine auxotrophs, *hisG52* and *hisC2*, were obtained from Dr. B. N. Ames; the purine auxotroph, *purH61*, was obtained from Dr. J. S. Gots; and the double mutant, *hisC2, purH61*, was constructed by transduction.

Cells were routinely grown in minimal medium,[16] with glucose at 0.5%, in 2 liters of culture medium in a 4-liter flask. The cultures were aerated vigorously in a New Brunswick rotary shaker at 37°.

In the case of the histidine auxotrophs, a sufficient amount of L-histidine was added to the cultures to support growth to an OD at 700 mμ of approximately 0.6 (5.8×10^8 cells per ml). Following histidine depletion, growth was supported by L-histidinol (Cyclo Chemical Co.). In the case of the wild-type organism, LT-2, derepression was effected by the addition of DL-2-thiazole alanine (Cyclo Chemical Co.), at a concentration of 0.1 mM, when the culture had reached an OD at 700 mμ of 0.6.[17]

In a typical experiment, aliquots were withdrawn from the culture periodically, so that 10 samples were collected before derepression and 10 samples were collected after derepression. Each sample was immediately mixed with ice and excess L-histidine in order to quickly halt the derepression process. After all samples had been collected, they were centrifuged. The pelleted cells were washed with 0.05 *M* Tris buffer, pH 8.0, containing histidine at a concentration of 0.1 mM, recentrifuged, and finally resuspended in 5 ml of 0.05 *M* Tris buffer, pH 8.0. Extracts were prepared from the suspensions with a French pressure cell (American Instrument Co.) at 6000 psi, clarified by centrifugation, and assayed immediately.

Protein was determined by the method of Lowry *et al.*[19] Assays for the enzymes corresponding to the first gene (*G*), third gene (*C*), fourth gene (*B*), sixth gene (*A*), and seventh gene (*F*) of the histidine operon were performed as previously described.[1, 2, 4, 5, 18, 20] Enzyme levels are expressed as the amount of activity per milligram of protein. Substrates were obtained as follows: L-histidinol phosphate was purchased from Cyclo Chemical Corp.; N-(5′-phospho-D-ribosylformimino)-5-amino-1-(5″-phosphoribosyl)-4-imidazolecarboxamide was synthesized enzymically[4] from 5-phosphoribosyl-1-pyrophosphate (P-L Biochemicals); and N-(5′-phospho-D-1′-ribulosylformimino)-5-amino-1-5″-phosphoribosyl)-4-imidazolecarboxamide was obtained as a gift from Dr. B. N. Ames.

Thiamine-HCl, L-serine, L-methionine, L-valine, L-histidine-HCl, and guanine were purchased

from Sigma Chemical Co.; thymine and adenine were purchased from Nutritional Biochemicals Corp.; sulfacetamide was a gift of Schering Corp.; and 2,4-diamino-5(3′, 4′, 5′trimethoxybenzyl)-pyrimidine (trimethoprim) was a gift of Dr. G. H. Hitchings of Burroughs Wellcome and Co.

Results and Discussion.—Over the past four years evidence from several laboratories has indicated that *N*-formylmethionine is involved in the initiation of polypeptide chains. In 1963, Waller showed that methionine is the most commonly occurring amino-terminal amino acid of *E. coli* protein[21] and soon thereafter, Marcker and Sanger demonstrated the existence of a species of tRNA specific for the incorporation of *N*-formylmethionine.[22] Subsequently, it was observed that *N*-formylmethionine was incorporated into the amino-terminal position of the coat proteins of RNA bacteriophages *in vitro*.[23, 24] More recently, two codons have been shown to specify *N*-formylmethionyl-tRNA when present at the 5′-terminus of synthetic polyribonucleotides.[25]

In a previous report from this laboratory it was concluded that phosphoribosyl-AIC, produced as a byproduct of the histidine pathway, is responsible for the phenomenon of sequential derepression.[15] The first step in the further metabolism of this compound requires the addition of a formyl group from N^{10}-formyltetrahydrofolate via a specific transformylase[6] (see Fig. 1). By creating an additional demand for N^{10}-formyltetrahydrofolate, this reaction might be expected to reduce the amount of formyl groups available for other reactions in the cell. Therefore, we considered the possibility that the mode of derepression of the histidine enzymes depends upon the relative availability of formyl groups for polypeptide chain initiation. According to this hypothesis, when the formylating capacity of the cell is adequate, translation of the polycistronic histidine message is initiated at the beginning of each cistron; when the formylating capacity of the cell is limiting, the translation of this message is initiated only at the operator end. Although these alternative ways of initiating translation of the polycistronic histidine message do not require that transcription and translation are coupled processes,[26, 27] they are consistent with this possibility.[28] Evidence in support of the hypothesis that the mode of derepression depends upon the relative availability of formyl groups for polypeptide chain initiation was obtained by examining: (1) the effect of a phosphoribosyl-AIC transformylase mutation in an organism that produces phosphoribosyl-AIC via the histidine pathway; (2) the effect of compounds which increase the formylating capacity in an organism which produces phosphoribosyl-AIC via the histidine pathway; and (3) the effect of compounds that limit the formylating capacity in an organism which does not produce phosphoribosyl-AIC via the histidine pathway.

The double mutant, *hisC2, purH61*, contains a mutation that prevents the conversion of imidazole acetol phosphate to histidinol phosphate (in the pathway for histidine biosynthesis) and a mutation that prevents the conversion of phosphoribosyl-AIC to formylphosphoribosyl-AIC (in the purine pathway) (see Fig. 1). The enzymes for histidine biosynthesis derepress sequentially in *hisC2*,[15] since this auxotroph produces phosphoribosyl-AIC via the histidine pathway. However, the phosphoribosyl-AIC produced as a byproduct of the histidine pathway in the *double* mutant cannot limit the formylating capacity of the cell, since the enzyme required for its conversion to formylphosphoribosyl-AIC is absent.[30, 31] In contrast to the sequential mode of derepression observed in *hisC2* (Fig. 2, part *A*), the enzymes for

histidine biosynthesis in *hisC2, purH61* derepressed simultaneously (Fig. 2, part *B*). It was previously suggested that the phosphoribosyl-AIC that is produced via the histidine pathway is responsible, directly or indirectly, for the phenomenon of sequential derepression of the histidine enzymes.[15] The elimination of sequential derepression by the transformylase mutation is consistent with the idea that phosphoribosyl-AIC exerts its effect on the mode of derepression by limiting the amount of N^{10}-formyltetrahydrofolate in the cell.

The role of formyl groups in determining the mode of derepression is further supported by the effect of compounds which increase the formylating capacity of the cell. The amino acids, L-serine and L-methionine, when added exogenously, would be expected to enrich the one-carbon pool in the cell, since they participate in one-carbon transfer reactions and require one-carbon addition for their own biosynthesis.[32] According to our hypothesis, increasing the size of the one-carbon pool (overcoming the limitation imposed by the phosphoribosyl-AIC produced via the histidine pathway), should allow chain initiation at multiple points along the poly-

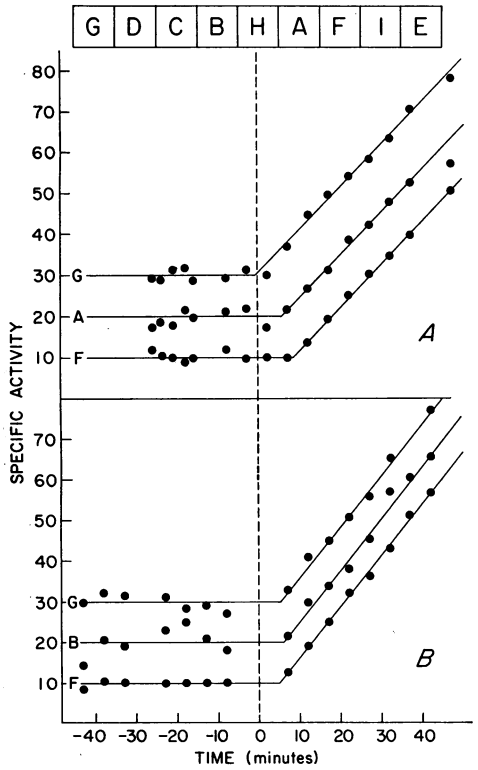

FIG. 2.—The influence of a phosphoribosyl-AIC transformylase mutation on the mode of derepression. Part *A* shows the kinetics of derepression in *hisC2*. Part *B* shows the kinetics of derepression in the double mutant, *hisC2,purH61*. For both of these experiments, the organisms were grown in the presence of adenine (5 μg/ml), guanine (5 μg/ml), and thiamine (1 μg/ml).[36]

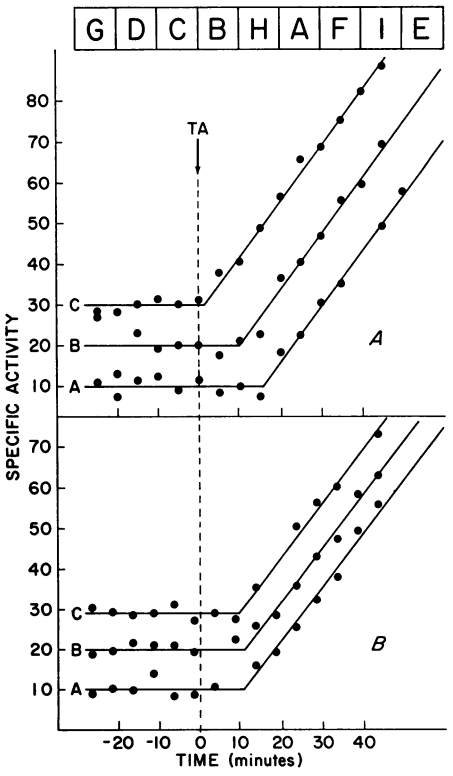

FIG. 3.—The influence of L-serine on the mode of derepression. Part *A* shows the kinetics of derepression in LT-2. Part *B* shows the kinetics of derepression in LT-2 grown in the presence of L-serine (80 μg/ml).[36]

cistronic histidine message, shifting the mode of derepression from sequential to simultaneous. In Figure 3 are shown the results of an experiment in which L-serine had been added to a culture of the wild-type organism 20 minutes before derepression of the histidine enzymes was effected with thiazole alanine. In the absence of L-serine, this organism is characterized by the sequential mode of derepression[15] (Fig. 3, part *A*), whereas in the presence of L-serine the mode of derepression was shifted to simultaneous (Fig. 3, part *B*). This shift in the mode of derepression was also observed when L-methionine was substituted for L-serine. In contrast, the amino acid, L-valine, which does not participate in one-carbon transfer reactions and does not require one-carbon addition for its own biosynthesis, had no effect on the mode of derepression.

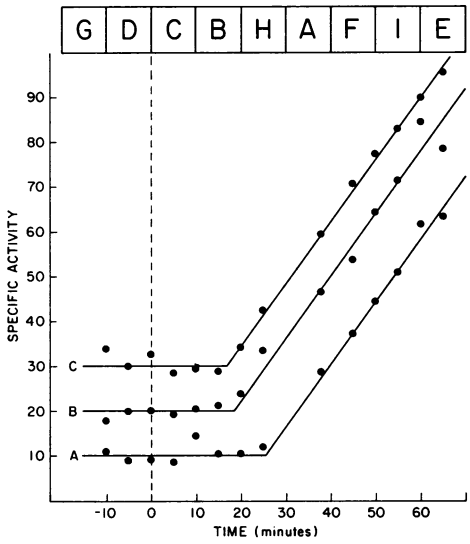

FIG. 4.—The kinetics of derepression in *hisG52* grown in the presence of sulfacetamide (2 mg/ml).[36, 37]

Since increasing the formylating capacity of the cell shifts the mode of derepression from sequential to simultaneous, we anticipated that decreasing the formylating capacity of the cell would shift the mode of derepression from simultaneous to sequential. N^1-acetylsulfanilamide, sodium salt (sulfacetamide), was used to partially inhibit the synthesis of folic acid.[33] According to our hypothesis, inhibition of folic acid synthesis should restrict initiation of polypeptide chains since the donor of formyl groups for methionyl-tRNA is N^{10}-formyltetrahydrofolate. For these experiments, cells were grown in the presence of sulfacetamide for at least 1.5 generations in order to deplete the folic acid pool. In order to avoid depletion of essential metabolites which require folic acid for their biosynthesis, thymine, L-serine, L-methionine, and adenine were provided in the culture medium. The results shown in Figure 4 demonstrate the effect of sulfacetamide in a histidine auxotroph previously characterized by the simultaneous mode of derepression.[15] As anticipated, the mode of derepression was shifted to sequential.

Formation of N^{10}-formyltetrahydrofolate requires the prior reduction of dihydrofolate to tetrahydrofolate, a reaction catalyzed by the enzyme, dihydrofolate reductase.[34] An inhibitor of this enzyme would decrease the formylating capacity of the cell.[38] Such an inhibitor would be expected to have the same effect as sulfacetamide, but this effect would be achieved by preventing the conversion of folic acid to N^{10}-tetrahydrofolate rather than by preventing the synthesis of folic acid itself. The dihydrofolate[39] reductase was inhibited by adding 2,4-diamino-5(3′,4′,5′trimethoxybenzyl)-pyridine (trimethoprim)(approximately 3 min prior to derepression) to a culture of a histidine auxotroph that had previously been characterized by the simultaneous mode of derepression.[15] Thymine, L-serine, L-methionine, and adenine were provided in the culture medium. As in the experiments with sulfacet-

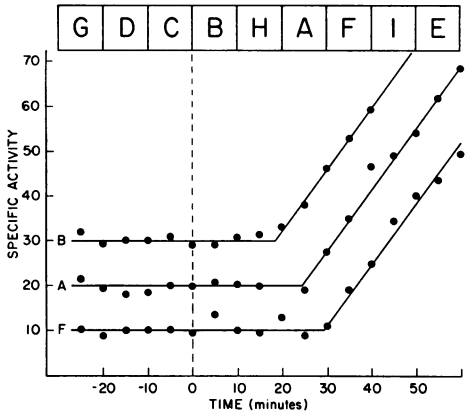

FIG. 5.—The influence of trimethoprim (2 μg/ml) on the mode of derepression in *hisG52*.[36,37]

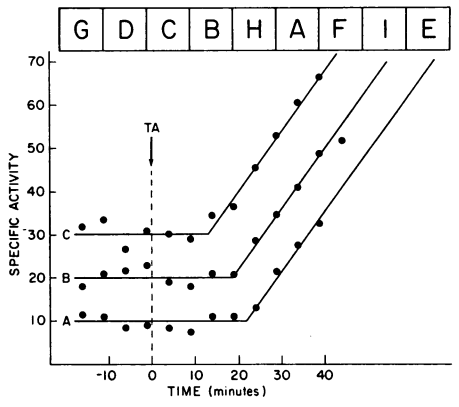

FIG. 6.—The influence of trimethoprim (5 μg/ml) on the effect of adenine, thymine, L-serine, and L-methionine on the mode of derepression in LT-2.[36, 37]

amide, the mode of derepression was shifted from simultaneous to sequential (Fig. 5).

As discussed above, L-serine, L-methionine, and adenine shift the mode of derepression from sequential to simultaneous. In order to support the conclusion that these compounds shift the mode of derepression by increasing the one-carbon pool of the cell, we attempted to demonstrate that their effect on derepression is prevented when *utilization* of one-carbon fragments is impaired. We therefore investigated the ability of trimethoprim to prevent the effect of these compounds on the mode of derepression. As shown in Figure 6, the latter compounds are not able to shift the mode of derepression from sequential to simultaneous in the presence of the inhibitor of dihydrofolate reductase.

The experiments reported here demonstrate that the manner in which the polycistronic message of the histidine operon is translated *in vivo* depends upon the capacity of the cell to initiate polypeptide chains. Specifically, it appears that the amount of N^{10}-formyltetrahydrofolate available (presumably for formylation of methionyl-tRNA) determines whether chain initiation occurs only at the operator end or at multiple sites along the polycistronic messenger-RNA.

In those mutants that synthesize phosphoribosyl-AIC via the histidine pathway, translation of the polycistronic histidine message is initiated only at the operator end, producing the sequential pattern of derepression, whereas in those mutants that do not synthesize phosphoribosyl-AIC via the histidine pathway, translation of this message is initiated at multiple points, producing the simultaneous pattern of derepression. The present study indicates that the manner in which phosphoribosyl-AIC produces the sequential pattern of derepression is indirect. It is the conversion of phosphoribosyl-AIC to formylphosphoribosyl-AIC that produces the sequential pattern of derepression by limiting the formylating capacity of the cell for polypeptide chain initiation. On the basis of the observation that a limitation of the formylating capacity of the cell produces the sequential mode of derepression, we conclude that when the formylating capacity is limiting, polypeptide chain initiation occurs preferentially at the operator end of the message. It is not known

whether this preference is due to the presence of a more efficient chain initiating codon at the operator end of the message than at internal positions or to other factors.

Since translation of the histidine message can be initiated at multiple sites only when formyl groups are not limiting, it appears that formyl groups are utilized in the initiation reactions at these sites. The simplest explanation for this utilization of formyl groups is that *N*-formylmethionyl-tRNA is required for polypeptide chain initiation at each cistron. Eisenstadt and Lengyel[40] showed that *N*-formylmethionyl-tRNA is required for amino acid incorporation into all the proteins programed by the polycistronic f2 RNA. The recent studies of Viñuela *et al.*[35] have shown that *N*-formylmethionine is the amino-terminal amino acid for all the proteins programed by the polycistronic MS2 RNA.

In vitro studies with synthetic polyribonucleotides have demonstrated that the codons which specify *N*-formylmethionyl-tRNA when present at the 5'-terminus do not do so when present at internal positions.[25] Therefore, it would appear that punctuation exists in the polycistronic histidine message *in vivo*, allowing such codons to specify *N*-formylmethionyl-tRNA despite their internal positions.

Note added in proof: Eisenstadt and Lengyel (personal communication) have observed that the addition of phosphoribosyl-AIC to an *in vitro* protein synthesizing system prevented amino acid incorporation into the histidine containing proteins programed by the f2 message. This effect could be overcome by the addition of *N*-formylmethionyl-tRNA.

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