Mutator and Antimutator Effects of the Bacteriophage P1 hot Gene Product

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The Hot (homolog of theta) protein of bacteriophage P1 can substitute for the Escherichia coli DNA polymerase III β subunit, as evidenced by its stabilizing effect on certain dnaQ mutants that carry an unstable polymerase III ε proofreading subunit (antimutator effect). Here, we show that Hot can also cause an increase in the mutability of various E. coli strains (mutator effect). The hot mutator effect differs from the one caused by the lack of HolE. Experiments using chimeric 0/HolE proteins containing various domains of Hot and HolE along with a series of point mutants show that both N- and C-terminal parts of each protein are important for stabilizing the ε subunit. In contrast, the N-terminal part of Hot appears uniquely responsible for its mutator activity.

The Escherichia coli chromosome is replicated with high efficiency and fidelity by DNA polymerase (Pol) III holoenzyme (HE), a large dimeric multisubunit enzyme complex that simultaneously copies the leading and lagging strands at the replication fork (for reviews, see references 15 and 24). HE contains two core polymerase subassemblies, each composed of three separate subunits, α, ε, and θ, which are tightly bound in the linear order α-ε-θ. The α subunit (dnaE gene product) (135 kDa) is the polymerase, while the ε subunit (dnaQ gene product) (28.5 kDa) is the 3′→5′ exonucleolytic activity that functions as a proofreader for replication errors.

The precise function of the θ subunit (holE gene product) (8 kDa) within the Pol III core is less clear. θ binds tightly to the ε subunit and does not seem to interact with the α subunit (2, 32). Strains lacking 0 (ΔholE strains) are viable, indicating that the subunit is not essential (31). On the other hand, our laboratory has shown that such deletion strains possess a mutator phenotype in mismatch repair-defective strains, suggesting that θ may have a positive effect on the accuracy of DNA replication, likely through its effect on the ε proofreading activity (33). This fidelity role is consistent with the increase in the ε exonuclease activity observed in an in vitro exonuclease assay in the presence of 0 (32). In addition, large effects of the deletion of 0 were observed in strains containing an impaired or unstable ε subunit (33). For example, the mutability of the temperature-sensitive dnaQ920 mutator strain was increased nearly 1,000-fold upon the loss of 0 (ΔholE strain). Based on these and other results, it was postulated (33) that the θ subunit fulfills a general stabilizing role for the intrinsically unstable ε subunit (7, 10, 12).

0 appears to be well preserved throughout the enterobacteria, suggestive of a meaningful role for the protein. Homologs of 0 have also been found, surprisingly, to be encoded by two conjugative plasmids as well as by bacteriophage P1 (3). In experiments with this P1 homolog produced by the P1 hot gene (homolog of theta) (22), we showed that the resulting Hot protein can substitute for 0 in certain dnaQ mutators such as dnaQ49, as it was capable of reducing the high mutability of a dnaQ49 holE strain (3). In fact, for dnaQ49, which carries a V96G mutation in ε (33), Hot appeared to be significantly more efficient than 0 in stabilizing this mutant. For other mutants, such as dnaQ920 (R56W), dnaQ923 (H66Y), and dnaQ924 (L171F), Hot proved as efficient as 0. In contrast, Hot was not able to substitute for the 0 function in dnaQ928 (G17S), indicating that the precise interactions of 0 and Hot with ε differ in certain details and that their effects may depend on the precise defect in ε (3).

The interaction between ε and 0 has also been pursued by structural studies (5–7, 11, 13, 16). Nuclear magnetic resonance (NMR) solution structures of both 0 and Hot have been obtained, revealing a largely superimposable three-part helical structure with unstructured N- and C-terminal segments (6, 26). Nevertheless, some small differences between the protein structures are apparent, as expected for two proteins that are approximately 50% identical (65% homologous). In addition, the biochemical behavior of Hot is slightly different from that of 0, as the purified protein appears to be more stable and better structured, allowing its solution structure to be determined in aqueous solution (6), whereas that of 0 required mixed alcohol-water solvents (26).

In the present report, we describe and investigate certain instances where the substitution of 0 by Hot produced, surprisingly, a mutator effect. This proved to be the case for the dnaQ930 (H89Y) mutator strain as well as the dnaQ+ (wild-type [wt]) strain. In addition, we made use of the sequence and structural homologies of the two proteins to create a number of chimeric protein molecules, which might permit a more precise definition of the subdomains of either protein responsible for the antimutator and mutator effects. The results indicate that the N-terminal domain of Hot, which appears unstructured in NMR solution spectra, is specifically responsible for the Hot mutator effect. We also show

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that Hot and θ compete for their incorporation into the Pol III holoenzyme.

**MATERIALS AND METHODS**

**Strains and media.** The *E. coli* strains used, along with information on their source or construction, are listed in Table 1. P1 transductions were performed using P1virA. The various *dnaQ* alleles (Table 1) were transduced using linkage (40%) with transposon *sae*-202::Tn10 as described previously (33). The sequenc-eholE as an internal primer. The resulting plasmids were analyzed by PCR and DNA sequencing. Sequencing primers were SeqHolE and SeqholELow (3). The sequencing also revealed, in addition to correct products, isolates containing

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The frequencies of rifampin-resistant mutants were calculated for 12 to 15 independent cultures for each strain, and the data were analyzed using Prism statistical analysis software (GraphPad). To validate the statistical significance of observed differences, the mutant frequency data were analyzed with the nonparametric Mann-Whitney test. An exact two-tailed P value was calculated for the tested data, and differences were determined to be statistically significant when the P value was <0.05.

RESULTS

Mutator and antimutator effects of P1 Hot. Our laboratory previously demonstrated that several dnaQ mutator strains carrying defects in the Pol III ε proofreading subunit are stabilized by θ (34). Their mutability, a reflection of their proofreading deficiency, was greatly enhanced in a ΔholE background. For example, the mutability of the dnaQ49 (V96G) allele was enhanced more than 1,000-fold by the lack of θ. In general, we noted a correlation between the recessive nature of dnaQ mutants, an indication of some structural impairment, and their sensitivity to the absence of θ. Subsequently, we showed that the bacteriophage P1 Hot protein, a putative θ homolog, was able to substitute for θ in several of these mutants, including dnaQ49 (3). Interestingly, for one other mutant, dnaQ924 (G178S), Hot appeared to be ineffective, although it was stabilized significantly (~100-fold) by θ (3). It was suggested that the interactions of θ and Hot with ε, although presumably similar, were not precisely identical in all respects.

In the present study, we have extended the comparative analysis of θ and Hot to one more additional dnaQ mutator mutant, dnaQ930 (H98Y) (33, 34). The H98Y defect in dnaQ930 is genetically dominant, indicative of a mostly catalytic proofreading deficiency rather than a structural one (33). In such a case, stabilization by θ would not be expected and indeed was not observed (34). The effect of Hot on dnaQ930 is shown in Fig. 1. In this experiment, we compared the effect of several dnaQ mutator alleles in three different genetic backgrounds: wild type (holE+), ΔholE, or ΔholE:hot. In the last case, the chromosomal holE gene encoding θ is replaced, precisely, by the P1 hot gene coding sequence so that Hot is expressed from the resident chromosomal E. coli holE promoter instead of θ (3).

The results for dnaQ49, dnaQ924, and dnaQ928 confirm previous observations (3): dnaQ49 (V96G) and dnaQ924 (L171F) are stabilized by both θ and Hot (with dnaQ49 more effectively stabilized by Hot than by θ), while the dnaQ928 (G178S) mutator is stabilized by θ only. Interestingly, the dnaQ930 (H98Y) strain displays a strong mutator phenotype when Hot is expressed. The Hot mutator effect is about 13-fold, as observed in several experiments, compared to the corresponding strains expressing or lacking θ. Thus, in addition to the established antimutator effects, P1 Hot is also capable of producing mutator effects.

To further investigate this mutator activity, we analyzed the effect of Hot in the corresponding dnaQ+ (proofreading-proficient) background. A modest (about twofold) but reproducible (and statistically significant) mutator activity was also observed in this case (Fig. 1). As effects on proofreading and DNA replication errors are often best evaluated in mismatch repair-deficient strains (due to the lack of correction of the replication errors), we also investigated the activity of Hot in a dnaQ+ mutL strain. Again, a consistent two- to threefold mutator effect was observed (Fig. 1). These results with the dnaQ+ and dnaQ930 strains indicate that although Hot can substitute for θ in many dnaQ mutants and in fact is even more efficient in stabilizing the dnaQ49 mutator, it is also capable of increasing the mutant frequency in E. coli.

Competition between θ and Hot. We made use of the hot mutator effect to investigate the possible competition between θ and Hot when they are present in the same cell. For this investigation, we used the mismatch repair-deficient mutL strains. High-level expression of θ or Hot is deleterious (3), but both proteins can be satisfactorily overproduced from the low-
copy-number plasmid pKO3 (3). Three plasmids, pKO3-holE (containing the holE gene), pKO3-hot (containing the hot gene), and the control plasmid pKO3-ΔholE (3) (Table 1) were transformed, separately, into three E. coli strains, holE\(^+\), ΔholE, and ΔholE:hot, and the effect on the frequency of rifampin-resistant mutants was evaluated (Fig. 2). In the ΔholE strain (first set), we reproduced the hot mutator effect shown in Fig. 1. In the wild-type (holE\(^+\)) strain (second set), expression of Hot from pKO3-hot also produced the mutator effect, despite the presence of holE in the cell. Finally, in the Hot-expressing (ΔholE:hot) strain (third set), overexpression of hot from pKO3-holE clearly reduced the Hot-induced mutator effect. Thus, these results indicate that holE and Hot can compete for incorporation into the Pol III holoenzyme.

**Mutator effects of ΔholE.** Previously, our laboratory reported that the loss of holE (ΔholE strain) causes a modest mutator effect in dnaQ\(^+\) strains (34). This was interpreted to indicate that holE exerts a positive effect on the \(ε\) proofreading activity even in the proofreading-proficient (dnaQ\(^+\)) background (34). This result is confirmed in the results for the dnaQ\(^+\) mutL\(^+\) strain (Fig. 1), although in this case, no obvious effect was apparent in the mutL derivative. Nevertheless, a ΔholE mutator effect in the mutL background was clearly observed in a slightly different strain, KA796 (34). Here, we made use of the KA796 series to compare the mutator effect(s) of ΔholE and ΔholE:hot. This background has the advantage that it permits the analysis of the specificities of mutations using the lac reversion system developed previously by Cupples and Miller (4). Specifically, KA796 was made ΔholE and ΔholE:hot, which was followed by an introduction of the series of F\(′\) (pro lacIE) episomes originally present in strains CC101 through CC106. The latter permit measurement, in parallel, of each of the six possible base pair substitutions at a specific site in the lacZ gene (4). The strains were also made mutL\(\text{mutL·::}Tn5\). The results for both Rif\(^+\) mutants and lac revertants are shown in Table 3. While both ΔholE and ΔholE:hot produce a similar 1.6-fold increase in the frequency of Rif\(^+\) mutants, their respective mutator effects are actually quite dissimilar, as viewed by the lac specificity data. For example, the lack of \(ε\) caused significant increases (indicated in boldface type in Table 3) for G · C → T · A, A · T → T · A, and A · G → C substitutions, consistent with our previous report (34). In contrast, the hot mutator did not increase the A · T → T · A transversions but instead increased the G · C → A · T transitions (1.8-fold) as well as the \(-1\) frameshifts in strain FC40 (two- to threefold). Strain FC40 is routinely used for studying adaptive (or postplating) mutagenesis (9), but here, colonies were counted at 48 h. (We also observed a reproducible two- to threefold increase in postplating mutations in the case of the hot\(^+\) derivative but not in the ΔholE strain [data not shown].) Thus, while both Hot and the lack of \(ε\) produce a mutator effect, their specificities and, by implication, the precise mechanisms by which these effects are generated must be different.

**0-Hot chimeric proteins.** The mutator effect exerted by the PI Hot protein presumably reflects certain differences in the \(ε\)-Hot interaction compared to the \(ε\)-\(θ\) interaction. In the simplest model, Hot might bind more strongly to \(ε\) than \(θ\), a possibility suggested by its stronger stabilization of dnaQ49 (Fig. 1) (3). Such stronger binding, while greatly stabilizing dnaQ49, might lead to some structural or functional impairment of the exonuclease, observable as a mutator effect in some other dnaQ alleles. In an expanded version of this model, the mutator/antimutator effects might be ascribable to separate (sub)interactions within the \(ε\)-Hot and \(ε\)-\(θ\) complexes. We have investigated the latter possibility by creating several 0-Hot chi-

![FIG. 2. Competition of \(θ\) and Hot for incorporation into the Pol III core. Shown are the effects of the overproduction of \(θ\) or Hot from low-copy-number plasmid pKO3 in a dnaQ\(^+\) strain containing the ΔholE, holE\(^+\), or ΔholE:hot genetic configuration. The mismatch repair-defective strains used were NR17119 (ΔholE), NR17120 (holE\(^+\)), and NR17121 (ΔholE:hot) (Table 1) containing the indicated pKO3 plasmids (Table 1). Cultures were grown at 30\(^\circ\)C in LB plus chloramphenicol, and the plates were likewise incubated at 30\(^\circ\)C. The frequencies of rifampin-resistant mutants were determined for 10 to 15 independent cultures for each strain, and the data were analyzed using Prism software (GraphPad). The graph shows the median values and interquartile ranges for the frequencies of rifampin-resistant mutants.](http://jb.asm.org/)}
meric proteins and assaying their effect on some of the dnaQ mutator alleles.

Figure 3, displays the amino acid alignment of θ and Hot, including the secondary structural elements, as defined by structural studies (6, 26). Overall, θ and Hot are approximately 48 to 53% identical (60 to 66% similar). These numbers increase to approximately 70% and 80%, respectively, when considering the structured crease to approximately 70% and 80%, respectively, when con-

FIG. 3. Amino acid alignment of E. coli θ and P1 Hot along with secondary structure elements determined from NMR spectra (6, 26). Note that the N terminus of Hot contains one extra residue relative to that of θ and that the numbering of the corresponding residues in the two proteins differs by 1. α, α helix, L, loop.

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FIG. 4. Effects of θ-Hot and Hot-θ chimeric proteins. The strains used were NR17117 (dnaQ49 ΔhotE), NR16320 (dnaQ23 ΔholE), and NR16329 (dnaQ30 θholE) (Table 1) containing one of five indicated plasmids (Table 1). Cultures were grown overnight at 30°C in LB plus chloramphenicol; plates were incubated at the same temperature as the cultures. Frequencies of rifampin-resistant colonies were determined for 10 independent cultures for each strain, and the data were analyzed using Prism software (GraphPad). The graph shows the median values and interquartile ranges for the frequencies of rifampin-resistant mutants. The x axis indicates, for each dnaQ allele, the five strains containing the following plasmids: pKO3ΔholE, pKO3ΔholE, pKO3ΔholE:hot, pKO3-θΔHot, and pKO3-HotΔθ. See the text for details.
temperature dependent (14, 34), and previous studies have shown that while both Hot and θ can stabilize this allele, Hot is more effective than θ, a difference that becomes more pronounced at higher temperatures (3). The present data reproduce these findings. The results further indicate that the HotΔ12θ protein behaves essentially like θ, while the θ11Hot protein behaves like Hot. These results suggest that while the N-terminal 11 to 12 residues of θ or Hot may have a role in stabilizing the DnaQ49 protein, they are not the primary determinants for the greater efficiency of Hot in this respect. Apparently, these determinants lie in the remainder of the respective proteins.

A most informative result was obtained for the dnaQ930 allele (Fig. 5C). Here, the mutator effect of Hot was strongly reproduced by the HotΔ12θ protein, indicating that the N-terminal 12 residues of Hot are primarily responsible for the Hot mutator activity. A weaker mutator effect was also seen for the θ11Hot protein, suggesting that some other structures within Hot might also be relevant for the Hot mutator effect for dnaQ930.

Further corroborative insights were obtained from the experiment with the dnaQ923 mutant (Fig. 5B). While, ostensibly, θ and Hot are similarly active in stabilizing this dnaQ allele, the two chimeric proteins clearly reveal a split phenotype. The HotΔ12θ chimera increased the dnaQ923 mutant frequency up to threefold in comparison to either θ or Hot, while the θ11Hot protein showed a three- to fivefold decrease. Although other explanations are possible, it appears that the N-terminal 11 to 12 residues of Hot are responsible for a mutator effect in both dnaQ923 and dnaQ930, but in the dnaQ923 mutant, this mutator effect is compensated for by a simultaneous antimutator effect of the remaining C-terminal part of Hot. As dnaQ930 does not require any stabilization by either θ or Hot, this second effect is not observed for this mutant.

Effects of point mutants in chimeric proteins. During the creation of pKO3-θ11Hot and pKO3-HotΔ12θ plasmids, we also obtained (presumably through PCR amplification errors) several mutants carrying amino acid substitutions. Several of these mutants were tested in parallel with the plasmids described above. The results are listed in Table 4.

For the HotΔ12θ protein, two substitution mutants were obtained in the N-terminal Hot region. The Y2H and A7T mutations significantly increased the mutability of the dnaQ49 strain, indicating that this N-terminal portion of Hot is important for the stabilization of the DnaQ49 protein. Y2H also increased the mutability of dnaQ923, consistent with the requirement of this allele for stabilization by Hot or θ. Y2H did not affect the HotΔ12θ mutator effect on dnaQ930, consistent with the notion that dnaQ930 does not require stabilization by

![FIG. 5. Effects of the θ11Hot and HotΔ12θ chimeric proteins. The three panels show the effect of the θ11Hot and HotΔ12θ chimeric proteins along with the control proteins on the mutability of dnaQ49 (A), dnaQ923 (B), and dnaQ930 (C). The strains used were NR17117 (dnaQ49 ΔholE), NR16320 (dnaQ923 ΔholE), and NR16329 (dnaQ930 ΔholE) containing various plasmids as indicated along the x axis: pKO3-ΔholE, pKO3-holE, pKO3-hot, pKO3-HotΔ12θ, or pKO3-θ11Hot. The cultures were grown in LB with chloramphenicol at 30°C and 35°C for the dnaQ49 strains (A) and at 30°C for the dnaQ923 and dnaQ930 strains (B and C). Plates were incubated at the same temperature as the cultures. The frequencies of rifampin-resistant colonies were determined for 8 to 12 independent cultures for each strain, and the data were analyzed using Prism software (GraphPad). The graph shows the median values and interquartile ranges for the frequencies of rifampin-resistant mutants.](http://jb.asm.org/)

![TABLE 4. Effect of point mutants in HotΔ12θ and θ11Hot chimeric proteins on the mutability of dnaQ49, dnaQ923, and dnaQ930 mutants](http://jb.asm.org/)
Hot or θ. Interestingly, the A7T mutation lowered the mutability of both dnaQ923 and dnaQ930 and in fact nearly completely abolished the mutator effect of Hot₁₂θ on both alleles. These results are fully consistent with our proposal that the N-terminal 12 residues of Hot are responsible for the Hot mutator effect.

Two mutations, N17H and W51C, were obtained in the θ-specific part of Hot₁₂θ. The N17H defect was generally deleterious for dnaQ49 and, especially, dnaQ923, increasing the mutation frequency while not affecting the mutability of dnaQ930. These results are consistent with a stabilizing role of the C-terminal part. In contrast, W51C likely impairs the overall integrity of the protein, as the resulting hybrid lacked the ability to stabilize (dnaQ49 and dnaQ923) and greatly reduced the mutator effect on dnaQ930. W51 in θ and the corresponding Y52 in Hot likely occupy a critical position within helix α₃, as discussed previously (26).

The amino acid substitutions in the reciprocal θ₁₂Hot protein are less informative. The D9Y and V17I mutations are largely neutral, whereas F53S resembles the case of W51C as discussed above, reducing both the stabilizing effect on dnaQ49 and dnaQ923 and the mutator effect on dnaQ930. Thus, F53S likely represents a structurally impaired protein that has lost most of its capacity to interact with ε.

**DISCUSSION**

The current results provide new evidence for the importance of the ε-θ interaction within the Pol III core in determining the fidelity of replication in E. coli. Previously, we demonstrated that the lack of the θ subunit caused a mutator effect in wild-type cells and in several proofreading-impaired dnaQ mutants (34). This strongly suggested a stabilizing role for the θ subunit, presumably keeping ε in a structural conformation favorable for proofreading. Currently, we show that Hot, the θ₁₂Hot homolog of θ that is capable of substituting for θ in this stabilizing role, can also generate a mutator effect, thus providing a further indication of the importance of the ε-θ (or ε-Hot) interaction in the optimal functioning of the proofreading activity. Specifically, the present results indicate that the extreme N-terminal residues (residues 1 to 12) of Hot are responsible for this mutator activity. In the published structure of Hot (or θ), this segment is not observed, presumably because it is poorly structured or conformationally active (6, 26). However, it is very likely that this segment plays an important role in the interaction with the ε subunit. The isolation of the A7T mutant in the Hot₁₂θ chimera, showing abolishment of the mutator effect, further supports the importance of this segment for the ε-Hot interaction. Further structural studies on ε-Hot or ε-θ complexes may shed light on this important aspect.

**Different mutational specificities for holE and holE:hot.** It is interesting that both the lack of θ and the substitution of θ by Hot lead to a very similar mutator phenotype when assayed by the frequency of rifampin-resistant mutants. However, more careful analysis using a series of defined lac reversions shows that the mutator effects are clearly distinct. If both effects result simply from the impairment of ε proofreading, one must assume that different base-base mismatches are differentially sensitive to the two modes of proofreading disturbance. As exonucleolytic proofreading involves multiple steps, including the conformational changes associated with the transfer of the terminal mismatch from the polymerase active site to the exonuclease site, such differential effects should be considered. Alternatively, the differential specificities could reflect a more complicated mode of mutation production in ΔholE versus ΔholE:hot strains. For example, accessory DNA polymerases, such as Pol II or Pol IV, have been shown to be involved in the production of mutations, particularly when Pol III has difficulty extending certain terminal mispairs (1, 17). Possibly, HE without θ and HE containing Hot behave differently with respect to this phenomenon of polymerase trafficking. This will be an interesting area for further studies. Finally, a phylogenetic tree relating P1 Hot to other sequenced θ homologs clearly suggests that Hot is more closely related to homologs from more distant species such as Klebsiella than to θ itself (3). Thus, Hot is likely optimized for interaction with the ε subunit from other enterobacterial species, possibly explaining the observed mutator effect.

**The role of hot for P1.** A noteworthy aspect of the present work is the demonstration of direct competition between θ and Hot (Fig. 2). While we have not demonstrated a direct incorporation of Hot into the Pol III HE, the straightforward interpretation is that Pol III HE, when replicating the E. coli chromosome, contains Hot at least part of the time in the mixed holE:hot experiments and completely in the case of the ΔholE strain. This is, to our knowledge, the first demonstration of such a heterologous substitution in the Pol III HE. Such substitutions may also occur in P1-infected cells or in lysogens carrying the P1 prophage. These conclusions are relevant for the question as to why P1 carries, specifically, a gene for a θ homolog but no homolog for any other Pol III accessory subunit.

One possibility is that the increased mutation rate resulting from the incorporation of Hot in the HE is beneficial to the phage. As optimal mutation rates are proportional to genome size in DNA-based microbes (8), P1, based on its smaller genome, might tolerate and benefit from an increased mutation rate. Alternatively, the beneficial effect of Hot may be derived from an increased efficiency of replication. As the number of Pol III HE molecules per cell is very limited (23, 25), phage replication would probably benefit from an increase in their number. As ε is an intrinsically unstable protein (7, 10, 12) and θ and Hot stabilize ε, increased amounts of θ or Hot may result in increased amounts of the Pol III core and, ultimately, HE. The precise order of assembly of HE and its rate-limiting steps are unknown, and it might be worthwhile to study this under conditions of increased θ or Hot protein. Another interesting question is in which stage of the P1 life cycle the hot gene product is expressed or active. The gene has been classified as a “late” gene based on its predicted promoter structure (18, 19, 22), suggesting that it might be produced primarily late in the lytic cycle, when DNA replication has ceased and packaging ensues. It is hard to envision a role for a replication protein at this late stage, and this issue has to be investigated further, including expression of hot during lysogeny. Preliminary results from our laboratory (our unpublished data) have indicated that hot is expressed at least from the P1 prophage (lysogenic state).

In future studies, we will attempt to address the issue of the role of Hot in the P1 life cycle by investigating the properties
of a phage deleted for Hot as well as the important question of the timing of Hot expression as either a later or early gene.

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