Roles of Histidine 784 and Tyrosine 639 in Ribose Discrimination by T7 RNA Polymerase†

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ABSTRACT: On the basis of their recently described T7 RNA polymerase−T7 promoter crystal structure, Cheetham et al. [(1999) *Nature 399*, 80] propose that discrimination of the hydrogen bonding character of the elongating NTP ribose 2′-substituent involves a hydrogen bond to histidine 784. This would contradict a previous conclusion, based on the characterization of mutant RNAPs, that discrimination of the hydrogen bonding character of the ribose 2′-substituent depends solely on the hydroxyl group of tyrosine 639. To resolve this point, we prepared and characterized histidine 784 point mutants. We find that while these mutations reduce the activity of the polymerase, they do not significantly reduce the level of ribose discrimination. Furthermore, a mutant with alanine at position 784 preferentially utilizes NTPs with 2′-substituents capable of acting as hydrogen bond donors or acceptors (2′-OH and 2′-NH_2) over NTPs with substituents that lack such properties (2′-F and 2′-H). In contrast, mutation of tyrosine 639 to phenylalanine eliminates discrimination of ribose 2′-group hydrogen bonding character. The effects on ribose discrimination of mutating tyrosine 639 to phenylalanine are independent of the side chain at position 784. These results indicate that histidine 784 is not involved in discrimination of the ribose 2′-group of the elongating NTP. The ability of T7RNAP tyrosine 639, which is conserved in both RNA and DNA polymerases, to select for rNTPs appears to be due to the fact that in RNAPs this tyrosine is available to hydrogen bond to the ribose 2′-OH, while in DNAPs it is hydrogen bonded to a glutamic acid.

Nucleic acid polymerases require mechanisms for discriminating the ribose structure of the triphosphate so as to specify whether DNA or RNA will be the product of polymerization. Since a hydroxyl group is larger than a hydrogen substituent, selective utilization of 2′-deoxy substrates can take advantage of a steric clash between an amino acid side chain and the ribose 2′-OH. Such a mechanism is used by both DNAP[1] I and MMLV RT, where it has been shown that replacement of a single amino acid by a residue with a smaller side chain relieves a steric clash with the 2′-OH group of a bound rNTP and allows these enzymes to efficiently incorporate rNMPs (*1*−*3*). RNAPs must use a different mechanism, the most apparent being taking advantage of the hydrogen bonding potential of the 2′-OH group. On the basis of the recently described structure of a T7RNAP−T7 promoter complex, Cheetham et al. propose that T7RNAP H784 contributes to selective utilization of rNTPs by making a hydrogen bond to the ribose 2′-OH group (*4*). This is a surprising conclusion because previous studies have shown that mutation of T7RNAP tyrosine 639 to phenylalanine greatly enhances utilization of dNTPs via a mechanism that involves loss of discrimination of the H-bonding potential of the 2′-substituent (*5*−*7*). Though it is conceivable that discrimination of the ribose 2′-group could depend on two hydrogen bonds (one from the hydroxyl group of Y639 and one from H784), this is difficult to reconcile with the observation that the Y639F mutation reduces the catalytic specificity for rNTPs versus dNTPs [($k_{cat,rNTP}$/$K_{m,rNTP}$)/($k_{cat,dNTP}$/$K_{m,dNTP}$)] to an average value of 4.1 when Mg^{2+} is the catalytic cation, or to 1.7 when Mn^{2+} is used (*7*). For some rNTPs, the reduction in catalytic specificity is even greater. The catalytic specificity of Y639F for rGTP versus dGTP, for example, is only 2.0 in Mg^{2+} buffer and 1.2 in Mn^{2+} buffer. Utilization of 2′-F-NTPs, which also lack the H-bonding potential of a 2′-OH but exhibit a ribose pucker similar to those of rNTPs, is even more efficient than utilization of 2′-H- or 2′-NH_2-NTPs (*6*). Thus, the Y639F mutation virtually eliminates discrimination of the H-bonding character of the ribose 2′-substituent. However, if it were true that this mutation removes only one of two hydrogen bonding interactions involved in 2′-group discrimination, we would expect significant residual discrimination in this mutant.

Since structure−function studies indicate that T7 RNAP discrimination of the H-bonding character of the ribose 2′-group involves only the hydroxyl of Y639, but a recent crystallographic study concludes that H784 is involved, we prepared several H784 point mutants and characterized the

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[1] Abbreviations: RNAP, RNA polymerase; DNAP, DNA polymerase; RT, reverse transcriptase; MMLV, Moloney murine leukemia virus; dNTP, deoxyribonucleoside triphosphate; rNTP or NTP, ribonucleoside triphosphate; ddNTP, dideoxyribonucleoside triphosphate; Y or Tyr, tyrosine; V or Val, valine; T or Thr, threonine; F or Phe, phenylalanine; A or Ala, alanine; Q or Gln, glutamine.

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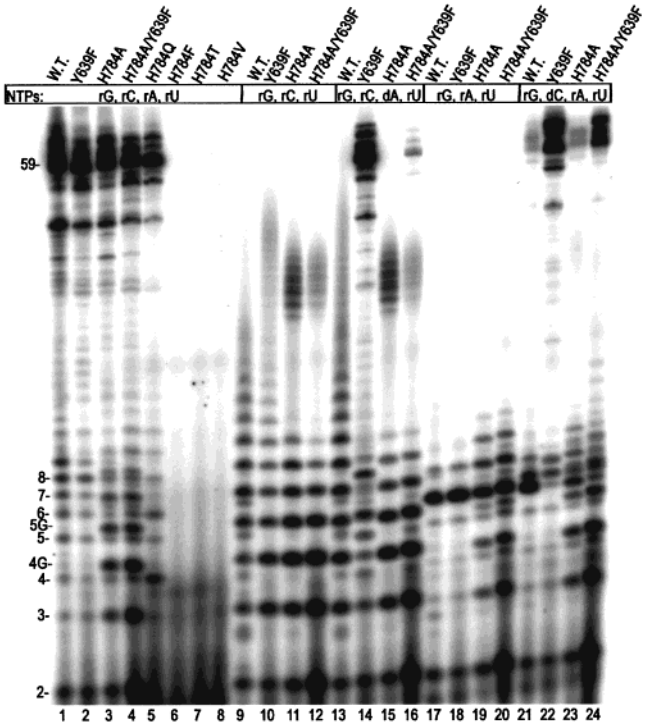

FIGURE 1: Transcription reactions run with *Hin*dIII-cut pT75 and the indicated RNAPs, rNTPs, and dNTPs. A 59-base runoff transcript is generated from initiation at the single class III T7 promoter present on this template. The sizes of the abortive (2−8mer) transcripts are indicated as are the four- and five-base "oligo-G" transcripts which are generated via transcript slippage during initiation (*17*). The longer extensions obtained with H784A or H784A/Y639F in lanes 19 and 20, respectively, may reflect higher levels of misincorporation by these mutants (see the text).

Table 1: Catalytic Specificity of Wild-Type and Mutant T7 RNAPs for rNTPs vs dNTPs[a]

	rGTP/dGTP	rATP/dATP	rUTP/dTTP	rCTP/dCTP
wild-type	68 ± 14	140 ± 15	39 ± 6.4	110 ± 18
H784A	51 ± 8	95 ± 12	31 ± 4.7	85 ± 7.2
Y639F	2.1 ± 0.4	7.1 ± 2.0	1.8 ± 0.1	5.2 ± 1.3
H784A/Y639F	1.7 ± 0.4	5.9 ± 0.7	1.6 ± 0.3	4.6 ± 0.3

[a] Values ± the standard error ($n = 3$).

catalytic and ribose discrimination properties of an active H784A mutant.

MATERIALS AND METHODS

Mutant construction, enzyme expression and purification, and nucleic acid preparation were as described previously (*6*). Measurement of the activity of the enzymes in runoff transcription reactions with rNTP and dNTP substrates (Figure 1) was as described previously (*5*). Determination of the catalytic specificity of the enzymes for rNTPs versus dNTPs (Table 1) was carried out as described previously (*7*), by measuring the rates of incorporation of a single α-^{32}P-labeled rNTP or dNTP in reactions in which all four rNTPs were present at a concentration of 0.5 mM, and a single α-^{32}P-labeled rNTP or dNTP was added to a final concentration of 33 nM. The ratio of the rate of percent incorporation of the radioactive rNTP relative to that of the radioactive dNTP in such assays has been previously shown to equal $(k_{cat,rNTP}/K_{m,rNTP})/(k_{cat,dNTP}/K_{m,dNTP})$. Measurement of the rates of dinucleotide synthesis (Table 2) was achieved as described previously (*8*). Measurement of the rates of 6mer synthesis (Table 2) was achieved by incubating RNAP (3.3×10^{-7} M) with supercoiled pT75 (1×10^{-7} M) in 25 μL reaction mixtures for 10 min at 37 °C in transcription buffer [40 mM Tris-HCl (pH 8.0), 5 mM DTT, and 10 mM $MgCl_2$]. Reactions were initiated by adding 0.5 mM GTP and ATP and 2.5 μCi of [α-^{32}P]GTP. Reaction aliquots taken at 2, 4, 8, 16, and 32 min were quenched and analyzed as described previously (*8*). Measurement of incorporation rates in single-round transcription assays (Table 2) was achieved by incubating RNAP and supercoiled pT75 at concentrations of 3.3×10^{-8} and 1×10^{-8} M, respectively, in transcription buffer for 10 min at room temperature, followed by addition of 0.5 mM GTP, ATP, and CTP to form halted elongation complexes. After incubation for a further 5 min, 0.5 mM UTP, 2.5 μCi of [α-^{32}P]UTP, and 1 mg/mL heparin (to limit reinitiation) were added. Reaction aliquots were then taken at 1, 2, 4, 8, and 16 min and quenched, and the extent of incorporation was measured by DE81 retention as described previously (*6*). Measurement of the activity of the enzymes in reactions with 2′-modified NTPs (Figure 2) was carried out by incubating RNAPs and supercoiled pT75 at concentrations of 3.3×10^{-8} and 1×10^{-8} M, respectively, in transcription buffer for 10 min at room temperature. Reactions were initiated by adding 0.5 mM GTP, CTP, and UTP, 0.13 μM [α-^{32}P]GTP (800 mCi/mM), and either rATP, dATP, 2′-F-ATP, or 2′-NH_2-ATP (all at 0.5 mM); aliquots were taken at 1, 2, 4, 8, and 16 min and quenched and analyzed as described previously (*6*).

Table 2: Rates of NMP Incorporation during Elongation and Rates of Synthesis of Two- and Six-Base Transcripts in Abortive Initiation Assays

	2mer[a]	6mer[a]	incorporation[b]
wild-type	1.3 ± 0.3 s^{-1}	6.1 ± 1.8 min^{-1}	30 ± 10 nucleotides s^{-1}
H784A	0.7 ± 0.2 s^{-1}	4.0 ± 0.9 min^{-1}	14 ± 3 nucleotides s^{-1}
Y639F	0.9 ± 0.3 s^{-1}	5.4 ± 0.1 min^{-1}	27 ± 3 nucleotides s^{-1}
H784A/Y639F	1.2 ± 0.3 s^{-1}	2.6 ± 0.4 min^{-1}	12 ± 4 nucleotides s^{-1}

[a] Values ± the standard error ($n = 3$). Rates of synthesis of 2- or 6mer molecules which incorporate a correction for the number of labeling NMPs incorporated into each molecule. [b] Rates of nucleotide incorporation during transcript elongation.

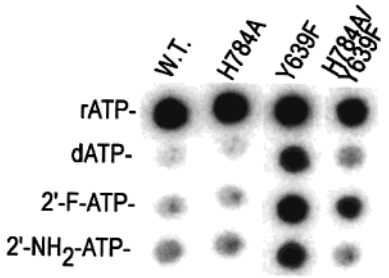

FIGURE 2: Incorporation of NMP into long transcripts with the indicated RNAPs measured by DE81 retention in transcription assays containing rCTP, rUTP, rGTP, [α-^{32}P]rGTP, and either rATP, dATP, 2′-F-ATP, or 2′-NH_2-ATP, as indicated. The relative rates of incorporation in reactions with 2′-OH-ATP, 2′-H-ATP, 2′-F-ATP, and 2′-NH_2-ATP (2′-OH:2′-H:2′-F:2′NH_2) for the enzymes were as follows: wild type, 100:1.4:2.1:5.4; Y639F, 100:18:29:16; H784A, 100:1.2:2.5:4.7; and H784A/Y639F, 100:8.1:47:6.8 (activity in the reaction with rATP was assigned a value of 100).

RESULTS

Histidine 784 is invariant in the phage RNAPs (*9*), suggesting that it is important for RNAP function. We were therefore concerned that mutation of this side chain might result in an inactive enzyme, so we made five different mutants to increase the likelihood of obtaining at least one active enzyme that would allow us to evaluate the role of H784 in ribose discrimination. To limit steric clashes, substitutions with smaller side chains of different polarity (H784V, H784A, and H784T) were introduced. H784F was constructed so as to maintain a planar ring system similar in size to the imidazole ring at this position, and H784Q was generated so as to retain a side chain nitrogen group and also because a glutamine is conserved at this position in the homologous class I DNA polymerases (*10*).

Runoff transcription assays revealed that H784A and H784Q were active (Figure 1, lanes 3 and 5) but that the H784T, -V, or -F enzymes were not (Figure 1, lanes 6−8). To determine whether the loss of the histidine side chain in the H784A mutant would result in the loss of ribose discrimination, we carried out reactions in which rATP was substituted with dATP (Figure 1, lanes 13−16) or rCTP was substituted with dCTP (lanes 21−24). Replacement of either rATP or rCTP with its deoxy counterpart reduced the level of runoff transcription by either the wild-type or H784A enzymes by >100-fold. The Y639F mutant, which has been previously shown to efficiently utilize dNTPs, displayed similar levels of runoff transcription in the four-rNTP, dATP + three-rNTP, and dCTP + three-rNTP reactions (lanes 2, 14, and 22, respectively). The H784A/Y639F double mutant also displayed enhanced utilization of dNTPs when compared to the wild-type or H784A enzymes, though its activity in the reactions with dNTPs was reduced relative to that of the Y639F single mutant.

In the dNTP-substituted reactions shown in Figure 1, the enzymes must incorporate dNMPs during initial transcription (at +4 and +6 or at +7 and +8 with dATP or dCTP, respectively) if they are to synthesize a runoff transcript. Such an assay might not reveal a mutation which has reduced ribose specificity because initial transcription is poorly processive, and has been shown to be extremely sensitive to even modest reductions in catalytic rates and decreased efficiency of utilization of noncanonical substrates (*11*−*13*). Conceivably, a mutant with reduced ribose specificity which is also less active might not be able to efficiently incorporate dNMPs during initial transcription, but might do so during elongation. To test this, we carried out reactions in the presence of all four rNTPs but in which either a [^{32}P]rNTP or a [^{32}P]dNTP was present in trace amounts to label the transcripts. The ratio of the rates of incorporation of the [^{32}P]-rNTP and the [^{32}P]dNTP in these reactions corresponds to the catalytic specificity for rNTPs versus dNTPs [($k_{cat,rNTP}$/$K_{m,rNTP}$)/($k_{cat,dNTP}$/$K_{m,dNTP}$)], but since all four rNTPs are present and the dNTP is present in only trace amounts, initiation is not limited by a requirement for dNMP incorporation (*7*). Data for the catalytic specificities of the wild-type and mutant enzymes are presented in Table 1. Relative to that of the wild-type enzyme, the catalytic specificity of the Y639F and Y639F/H784A mutants for rNTPs versus dNTPs is reduced by an average of 24- and 28-fold, respectively, while that of the H784A mutant is reduced by 1.3-fold. To determine if the H784 mutants specifically retain the ability to discriminate the H-bonding character of the 2′-substituent, we measured their activity in reaction mixtures in which a single rNTP was replaced with either a dNTP, or an NTP with an amino group, or a fluorine, at the ribose 2′-position (Figure 2). In such reactions, the rank order preference of the wild-type enzyme for NTPs with different substituents is as follows: 2′-OH > 2′-NH_2 > 2′-F > 2′-H. This order reflects a preference for substituents that can act as hydrogen bond donors or acceptors, while the rank order preference of Y639F is as follows: 2′-OH > 2′-F > 2′-H > 2′-NH_2. The latter rank order may be determined by ribose pucker, and reveals that the H-bonding character of the 2′-substitutent is no longer the primary determinant of NTP preference (*6*). H784A displays a rank order preference for these substituents identical to that of the wild-type enzyme (Figure 2), while the H784A/Y639F double mutant displays a preference like that of Y639F.

To assess the effects of the loss of the H784 side chain on enzyme activity, we measured the steady-state rates of short transcript synthesis in abortive initiation assays in which transcript extension was limited to two or six bases (Table 2). Table 2 also presents data for the rates of NMP incorporation into long (DE81 retainable) transcripts in single-round transcription assays where supercoiled plasmids were used as templates. We also quantified the percentage of abortive transcripts in runoff transcription to assess the effects of the mutations on the efficiency of promoter clearance and processivity during initial transcription. For the four rNTP reactions presented in Figure 1, 53 and 42% of the initiated transcripts were extended to runoff transcripts with the wild-type and Y639F enzymes, respectively. For the H784A and H784A/Y639F enzymes, only 34 and 14%, respectively, of the initiated transcripts were extended to runoff transcripts. Thus, the H784A and, in particular, the double mutation significantly reduce the efficiency of promoter clearance and, as can be seen in lanes 3 and 4 of Figure 1, decrease the processivity and increase the extent of oligo-G synthesis during initial transcription. Interestingly, while the H784Q mutation shows increased termination at four and six bases (Figure 1, lane 5) and a consequent reduction in the percentage of initiated transcripts which are extended to form runoff transcripts (29%), it does not display the increases in the extent of oligo-G synthesis or the increases in the extent of 2- and 3mer synthesis which are typical of mutations that decrease the efficiency of promoter clearance (*11*, *12*).

DISCUSSION

Though histidine 784 is invariant in the phage and mitochondrial RNAP family, the loss of this side chain has only a modest effect on enzyme activity. The rate of nucleotide incorporation in a single-round transcription assay with H784A is reduced by only a factor of ∼2 relative to that of the wild-type enzyme (Table 2), and the rates of short transcript synthesis in abortive initiation assays are reduced by less than a factor of 2. However, the H784 mutations do exhibit a marked decrease in processivity during initial transcription and a consequent substantial increase in the proportion of abortive transcripts (Figure 1). It has previously been reported that mutations with modest effects on catalytic rates can have large effects on processivity during initial

transcription (*11*, *12*), and it is therefore possible that effects on the efficiency of promoter clearance can account for the conservation of this side chain. Alternatively, conservation of H784 may also reflect a role for this side chain in fidelity, as we have found that H784 mutants exhibit increased misincorporation rates (J. Huang et al., unpublished observations).

Since the loss of the H784 side chain does not eliminate enzyme activity, the loss of activity in the H784V, -T, or -F mutation is most likely due to structural perturbation due to a steric clash in the enzyme or the enzyme−template−substrate complex. This is simplest to understand for the H784F mutation, where the substitution replaces the histidine with a larger side chain. On the other hand, the H784V or -T mutation replaces the His side chain with a smaller group, and both of these side chains are also smaller than the Gln side chain in the active H784Q mutant. Since there is also no correlation between side chain polarity and activity (Q and A are active; V and T are not), it appears likely that it is the branching at the β-carbon of the Val and Thr side chains which sterically disrupts the enzyme's function.

The primary object of this study was to test the hypothesis that T7 RNAP H784 is involved in discriminating the character of the ribose 2′-substituent of the elongating NTP. Our results are inconsistent with this hypothesis. Though H784A shows a small reduction (~1.3-fold) in rNTP versus dNTP specificity relative to that of the wild-type enzyme, the reduction in specificity is much less than the ~25-fold reduction seen when tyrosine 639 is mutated to phenylalanine. The effect of the Y639F mutation is independent of the side chain at position 784; a similar reduction in specificity is observed in both the Y639F single and the Y639F/H784A double mutant.

The very small reduction in ribose specificity seen in the H784A mutant may reflect a nonspecific relaxation of active site substrate specificity, rather than a specific loss of discrimination of ribose 2′-group H-bonding character. This is consistent with the observation that the H784A mutant retains a rank order preference for ribose 2′-substituents identical to that of the wild-type enzyme (rNTP > 2′-NH_2-NTP > 2′-F-NTP > 2′-dNTP) and apparently based primarily on their H-bonding character, while the Y639F and H784A/Y639F enzymes display a distinct rank order preference (rNTP > 2′-F-NTP > 2′-dNTP > 2′-NH_2-NTP) which is apparently determined by ribose conformation or, possibly, by some other aspect of 2′-group character. A nonspecific relaxation of active site specificity would be consistent with the finding that the H784A mutation increases the level of misincorporation (Y. Huang et al., unpublished observations).

To attempt to reconcile the observation that mutation of H784 does not affect discrimination of ribose 2′-group H-bonding character with the conclusion reached by Cheetham et al. on the basis of their structural studies, we superimposed the T7RNAP−promoter complex structure on the structure of the homologous T7DNAP complexed with the primer, the template, and ddNTP (Figure 3). T7RNAP H784 aligned with T7DNAP Q615. T7DNAP Q615 does not interact with the ddNTP ribose but instead makes a hydrogen bond with the template base opposite the primer terminus (*14*). The corresponding Q754 in the homologous Taq DNAP−primer−template−ddCTP structure also interacts with the template strand opposite the primer terminus (*10*). Mutation of the corresponding Q849 in *Escherichia coli* DNAP I affects catalysis and gives rise to an antimutator phenotype but does not affect ribose discrimination (*15*, *16*). In the model shown in Figure 3, the distance from the ribose 2′-carbon to either nitrogen of T7RNAP H784 is 4.6 Å, while that to the oxygen of T7RNAP Y639 is 3.0 Å. These distances imply that Y639, rather than H784, would be better positioned to discriminate the ribose 2′-group. However, a role for T7RNAP Y639 in the preferential utilization of rNTPs is puzzling, since this residue is conserved in both RNA and DNA polymerases. How does the same side chain select for rNTPs in RNAPs but not DNAPs? In T7DNAP, steric exclusion of rNTP utilization involves the side chain of E480. In T7RNAP, a glycine is at this position, and this makes room for a ribose 2′-OH group. Examination of Figure 3 suggests that T7DNAP E480 may contribute to dNTP selectivity not only by sterically interfering with rNTP utilization but also by hydrogen bonding to Y530. The latter residue corresponds to T7RNAP Y639. This suggests that substitution of the glutamic acid with a smaller side chain not only makes room for a hydroxyl group but also removes the hydrogen bond to the tyrosine and therefore frees this tyrosine to hydrogen bond with the ribose 2′-OH. Since the hydrogen bond between T7RNAP Y639 and the ribose 2′-OH contributes to ground state binding (*6*), we might expect that substitution of the glutamic acid with a smaller side chain in a DNAP might actually result in tighter binding of rNTPs versus dNTPs. In fact, this has been found in DNAPI, where substitution of the corresponding E710 with alanine creates an enzyme with K_m values for rCTP and dCTP of 3.5 and 18 μM, respectively (*2*).

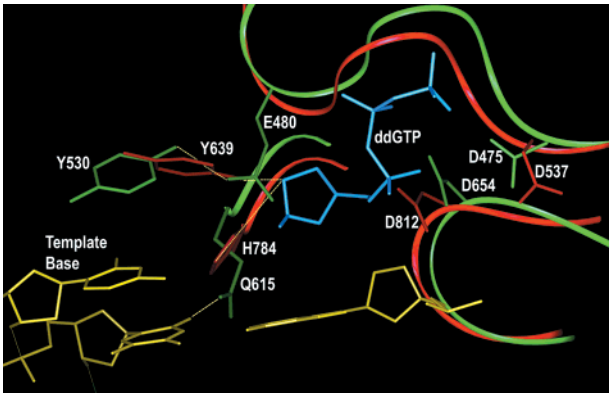

FIGURE 3: Superposition of the T7RNAP−promoter structure on the T7 DNAP−primer−template−ddNTP structure. Relevant side chains and part of the main chain for T7RNAP and T7DNAP are shown in red and green, respectively. The template and primer are yellow, and the ddGTP is light blue with the base removed for clarity. Superposition was carried out by using the program Insight II (MSI) to optimize the main chain alignment of T7DNAP helix O and β-strands 9, 12, and 13 with the corresponding elements of T7RNAP. The T7RNAP−promoter structure lacked side chain coordinates for Y639, so the preferred rotamer for this side chain was built into the model using the Biopolymer module of Insight II.

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