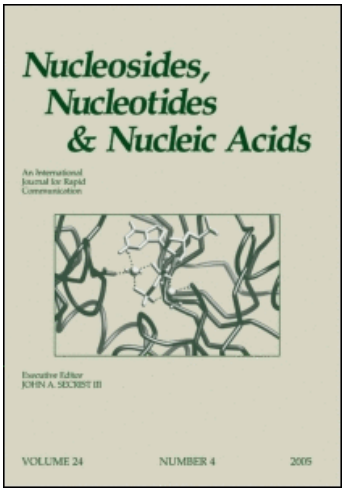

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Expanding the Catalytic Repertoire of Nucleic Acid Catalysts: Simultaneous Incorporation of Two Modified Deoxyribonucleoside Triphosphates Bearing Ammonium and Imidazolyl Functionalities.

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Abstract:

Two nucleoside triphosphates, a pyrimidine modified with an ammonium functionality and a purine modified with an imidazolyl functionality are compatible with all conditions for a combinatorial selection of nucleic-acid catalysts. We believe that this work is the first to demonstrate the potential for using not one but two modified nucleotides in tandem. The potential for an enriched catalytic repertoire is envisioned.

Introduction:

The discovery that single stranded polynucleotides carry both genetic information and catalytic potential has been exploited by selection procedures to develop aptamers with novel catalytic properties.[1] Catalysts have been selected for ligase[2], polymerase[3], and nuclease[4] activities wherein a properly oriented divalent metal cation and/or ammonium ion necessarily stabilizes the negatively charged pentacoordinate transition state phosphate. As the number of selected activities grows, what becomes apparent is that the kcat's of such catalysts usually do not compare very favorably with the kcat's of natural enzymes. Even in the few cases where aptamer and antibody catalysts have been selected to catalyze the same reaction, kcat's of the former are found to be inferior to those of the latter.[5,6] The reasons for the rather meager catalysis exhibited by nucleic acid derived catalysts have been the subject of numerous reviews.[7-9] In considering the general lack of chemical diversity of aptamers compared to proteins, perhaps the two

most striking deficiencies, at physiological pH, are the lack of a positive charge to stabilize anionic transition states and the lack of a functionality, which can accomplish general acid/base catalysis.[10] Such functionalities, conferred by lysines and histidines respectively, are found almost ubiquitously at the active sites of enzymes, particularly those, which catalyze reactions on phosphate.[11]

To begin to address this apparent catalytic shortcoming, herein we describe the potential for enhancing and enriching the chemical diversity of in-vitro selected DNA by simultaneously using two modified nucleoside triphosphates: one with an imidazolyl and one with an ammonium functionality. Although the use of a single modified nucleotide to alter and/or expand the recognition potential had been previously described, the potential for using not one but two synthetic nucleotides to introduce functionalities analogous to those found in enzyme active sites has never been reported.[12,13] The two nucleotides described here are: $dU^{aa}TP$ (5-(3-amino-allyl)-2'-deoxyribouridine-5'-triphosphate) and $dA^{im}TP$ (8-(2-(4-imidazolyl)ethylamino)-2'deoxyriboadenosine-5'-triphosphate).

For any selection involving a modified nucleoside triphosphate, four conditions must be satisfied. Firstly, the nucleoside triphosphate must retain its base-pairing capacity during the iterative cycling of information transfer. Secondly, the modified nucleoside triphosphate must be a substrate for at least one DNA polymerase. Thirdly, it must absolutely replace its unmodified counterpart at *every* position specified by the sequence. Fourthly, the single-stranded DNA containing the modified nucleotides must be able to serve as a template to be faithfully recopied into unmodified cDNA during each round of PCR amplification.[14] In order to satisfy the first condition, base modifications are usually introduced at the pyrimidine 5- or 6- position or at the purine 8-position so as not to alter the hydrogen bonding of Watson-Crick base-pairing.[15] In addition to the fact that such modifications must not alter base pairing, the nucleoside triphosphates must also be substrates for a polymerase. Generally, functionalities appended to the 5 position of uracil and the 8 position of adenine do not substantially impede uptake and incorporation by either DNA or RNA polymerase; bases modified with digoxigenin, biotin, alkylatable thiols, nitroxides, photoactive phenylazides, fluoresceine, and cyanine dyes have been successfully polymerized.[16-27] We recently reported the synthesis of $dA^{im}TP$, an analog that to the best of our knowledge had never been synthesized.[28] Although $dU^{aa}TP$ had already been shown to be a substrate for

DNA polymerase, and similar dATP analogs bearing larger appendages have been successfully incorporated, the substrate properties of $dA^{im}TP$ were unknown; particularly in conjunction with the readily available $dU^{aa}TP$ for simultaneous use in in-vitro selection.

In addition to the initial incorporation, the read-out upon recopying must be faithful with respect to sequence. This condition has not been previously addressed with respect to either modified nucleotide. It is with the eventual goal of selecting RNase mimics that herein we report that *both* $dU^{aa}TP$ and $dA^{im}TP$ can be faithfully incorporated by DNA polymerase and then recopied exponentially into non-modified cDNA such that all conditions are satisfied for their use in a combinatorial in-vitro selection scheme.

Materials and Methods:

Suppliers: The synthesis of $dA^{im}TP$ was previously described.[29] $dU^{aa}TP$ was obtained from Sigma-Aldrich. 100mM deoxyribonucleoside triphosphates and dideoxyribonucleoside triphosphates were obtained from Pharmacia. T4-polynucleotide kinase, Vent (exo-) DNA polymerase, HindIII, XbaI, and XmaI were obtained from New-England Biolabs. T7-Sequenase Version 2.0® and pyrophosphatase were obtained from Amersham Life Products-USB. All oligos numerically designated: #1 GTTTTCCCAGTCACGAC, #2CGAGCTCGGTACCCGGGG were obtained in pure form from Eurogentec.

Methods: For elongation of Oligo #1 (M13 universal sequencing primer) shown in Figure 1, the protocol was adapted from that contained in the USB Sequencing Kit.[30] In this case the concentrations of dGTP, dCTP, dATP, dTTP were held at 50μM. Polymerization of the modified DNA (mdDNA) occurred in the presence of $dU^{aa}TP$ (50μM) and $dA^{im}TP$ (88μM) as surrogates for dTTP and dATP respectively. Neither dATP nor dTTP was added. The concentration of the dideoxy-terminators was held at 12μM. 5'-labeled Oligo #1 that is complementary to M13 (-40) was labeled for the initial polymerization studies. DNA and mdDNA (modified DNA) were polymerized overnight by T7-Sequenase Version 2.0® in the presence of pyrophosphatase (approx. 1U each per reaction) in 40mM Tris-HCl pH 7.5, 20mM $MgCl_2$, 50mM NaCl, 5mM DTT, 2.5mM $MnCl_2$.

Restriction digests of mdDNA/DNA heteroduplexes: the polymerization products were desalted over a G50-Sephadex spin column and resuspended in the appropriate

restriction enzyme buffers for HindIII, XbaI, and XmaI. After overnight digestion, the samples were resolved by denaturing 8% PAGE.

For the PCR amplification studies, isolation of mdDNA was achieved by elution into 1-3 ml 7.5mM carbonate 0.1mM EDTA buffer. The sample was lyophilized to approximately 100µl and then precipitated with 4 volumes of acetone. It was then washed twice with 70% ethanol. A control elution was performed in the same region of a sham lane, which contained only primer and MI3. One sided (linear) high temperature amplification of an estimated 100fmol mdDNA was achieved using 2pmol 5'-labeled oligo #2 (>$2 \cdot 10^5$cpm/pmol) in a 50µl reaction mixture containing 20mM Tris-HCl, 10mM KCl, 10mM $(NH_4)_2SO_4$, 3mM $MgCl_2$, 0.1% Triton X-100, 200µM non-modified dNTPs and 5U Vent exo- DNA polymerase. The reaction was cycled 99 times from 53 to 75 to 95 degrees for 15 seconds per temperature step, 0-second ramp. The same reaction was performed on the contents eluted from the sham lane. Products were extracted with phenol-chloroform, precipitated with NaOAc and ethanol, subjected to Maxam-Gilbert chemical sequencing, and resolved by 15% denaturing urea-PAGE.

Results and Discussion:

The polymerization of mdDNA in the presence of two modified nucleoside triphosphates as surrogate substrates for dTTP and dATP is compared with the polymerization of unmodified DNA in Figure 1. Uninhibited polymerization is shown in lanes 1 and 6 while the dideoxy-terminated reactions follow in lanes 2-5 and 7-10. By and large, the terminated sequencing products in the modified cases parallel those in the unmodified case. Numerous attempts were made to improve gel resolution however the bands remained somewhat blurred, most likely due to an anticipated partial positive charge and additional steric bulk accumulating with increasing incorporations of the modified bases. Nevertheless, the sequence is fairly well resolved from position -10 (GGCCA etc.) to position +32 across 43 bases. A preceding sequence of 12 bases (from position -23 to -11), which initially demands the successful incorporation of 4 sequential $dA^{im}TP$'s, is too close to the primer to be resolved (data not shown).

Within the region of sequence resolution, certain biases are revealed in the modified case that are not apparent in the unmodified case. It is certainly possible that sequential incorporations of modified bases may be significantly disfavored. This would result in a comparatively stronger dideoxy-termination event in the second site compared to the first. Such is the case with the incorporation of two dU^{aa} bases at positions +4 and +5

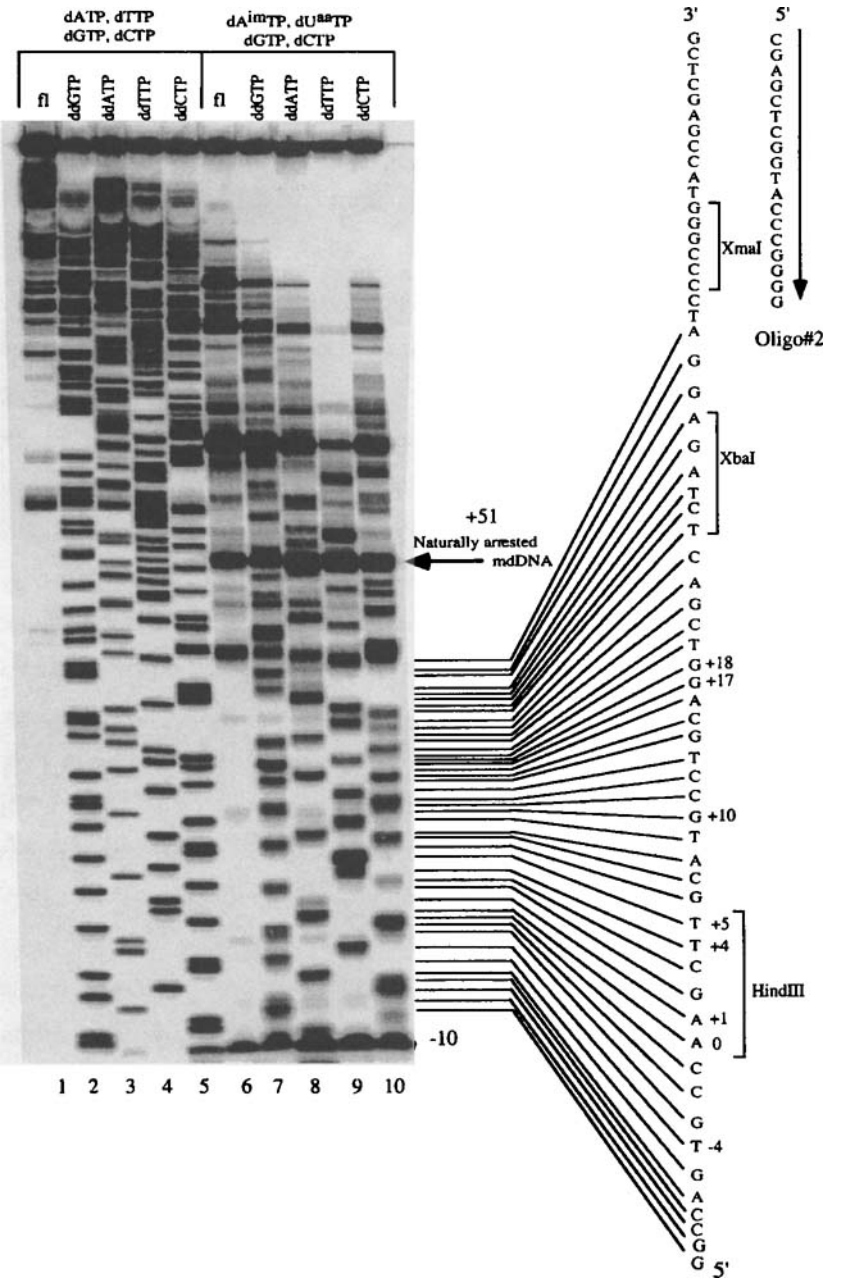

Figure 1: Elongation of universal sequencing primer by T7-Sequenase on M13 template. In the case of the unmodified dNTPs, each is 50μM. In the case of the modified dNTPs, each is at 50μM except for $dA^{im}TP$ which is 88μM. Lanes 1-5 are unmodified DNA while lanes 6-10 are modified. Lane 1: full length, Lane 2: ddGTP, Lane 3: ddATP, Lane 4: ddTTP, Lane 5: ddCTP, Lane 6: full length modified, Lane 7; ddGTP, Lane 8: ddATP, Lane 9: ddTTP, Lane 10: ddCTP. Sequence of the elongation product is shown at the right. The sequence of oligo #2 complementary to +51 is designated by arrow and its sequence is given.

where the incorporation of the first dU^{aa} seems to reduce the rate of incorporating a second thus favoring ddTTP termination. However no such phenomenon is seen around positions +6 to +11, where GCATGC are incorporated and terminated with the same relative efficiency at each site. Moreover, just the opposite effect is seen at positions 0 and +1, where it appears that the incorporation of the first $dA^{im}TP$ either reduces the Km for incorporation of a subsequent $dA^{im}TP$ (favoring a second incorporation) or raises the Km for the ddATP terminator. It is also possible that the preceding dU^{aa} at position -4 is responsible for this effect. Thus the kinetic and catalytic effects of sequential incorporation are not readily generalizable. In addition, the competition between the non-modified dGTP and dCTP and their respective dideoxy terminators is also affected in certain regions even though G and C are never modified. For example, the termination by ddGTP at positions -10 and -9 is not uniform whereas at positions +17 and +18, it is. Likewise, termination by ddCTP at position +3 is unusually weak compared to other positions. Thus, while terminations are non-uniform, there is no conclusive evidence that sequential incorporations of modified bases are necessarily disfavored. All in all, Figure 1 indicates in a global sense that modified bases give the same Sanger sequencing pattern that the non-modified bases do although with somewhat reduced resolution.

Following position +51, the sequence becomes less legible and several arrested bands occlude the resolution. Although the extension proceeds well beyond 100 bases, arrests do begin to accumulate. Although sequential incorporations of modified bases may indeed be responsible for arrests, they are also observed in lane 1 even with unmodified bases. In the presence of higher concentrations of modified dNTPs, these arrests were somewhat alleviated (data not shown). However, since the incorporation across 55 bases (-22 to +32) containing 8 A's and 9 T's proceeded without significant arrest, the nature of these arrests was not fully investigated, particularly because compression artifacts and the like are a known phenomenon. To fully understand the cause for arrest, a full kinetic analysis would be necessary. The complicated kinetics of incorporation, which can be highly dependent on the immediately preceding sequence, were not addressed at this juncture. We did attempt to examine incorporation using a partial complement of dNTPs. However, even using a partial complement of unmodified dNTPs, the high processivity and lack of proofreading activity of the polymerase gave rise to mis-incorporations.

Although some selections have used up to 100 random positions, most in-vitro selections involve only 40 degenerate positions, so while arrests might be seen with longer incorporations, the first 50 bases were extended without major inhibition. Certainly, the use of Im-dATP and AA-dUTP in conjunction with 100 degenerate positions may be less effective due to arrests which might be nevertheless be alleviated by increasing the dNTP concentration. However there's little reason to use 100 degenerate positions when 12-15 bases are sufficient to deliver highly competent catalysis.[31] The catalytic domains of Hammerhead and Hairpin ribozymes fall under 40 bases as well. In addition, a 200nmol degenerate synthesis will deliver a population ($1.2 \cdot 10^{17}$ total molecules) corresponding to *only* 28 degenerate positions ($7.2 \cdot 10^{16}$ combinations). Theoretical limits and practical findings would indicate that there's no reason to use more than 28 degenerate positions, let alone 100.

We felt that demonstrating viable incorporation of 8 $dU^{aa}TPs$ and 9 $dA^{im}TPs$ evenly distributed across approximately 50 bases would be sufficient to justify their eventual use in a combinatorial in-vitro selection scheme without performing an in-depth kinetic analysis on other templates such as homo-base tracts for two reasons. Firstly, since polymerases do not effectively polymerize along homo-base tracts, data from such templates would only be more confusing, not less.[32] Moreover, examining homo-base tracts would seem superfluous: when the number of degenerate positions exceeds 15, the fraction of all-A/T + all-A/C + all-G/T sequences is *vanishingly* small i.e. 0.00009 ($3 \cdot 2^{-15}$).

Secondly, since only two imidazolyl and two ammonium functionalities (His 12&119 and Lys 7&41) are found to be critical for maintaining RNaseA activity, selection of an analogous activity should be possible given that there seemed to be little problem in incorporating at least two of each modification.[33]

To further demonstrate the incorporation of the two nucleotides as well as begin to characterize some of the properties of mdDNA, we examined the sensitivity of the mdDNA/DNA heteroduplex to three restriction enzymes in Figure 2. Oligo #1 was elongated by Sequenase in the presence of either non-modified or modified dNTPs. The resulting duplex DNA was then digested with HindIII, XbaI, and XmaI, which cleave at AAGCTT, TCTAGA, and CCCGGG respectively. Lanes 3,5, and 7 are the non-modified controls demonstrating nearly quantitative cleavage (some star activity in the case of XmaI was also seen). Lanes 4,6, and 8 are the same digests in the presence of

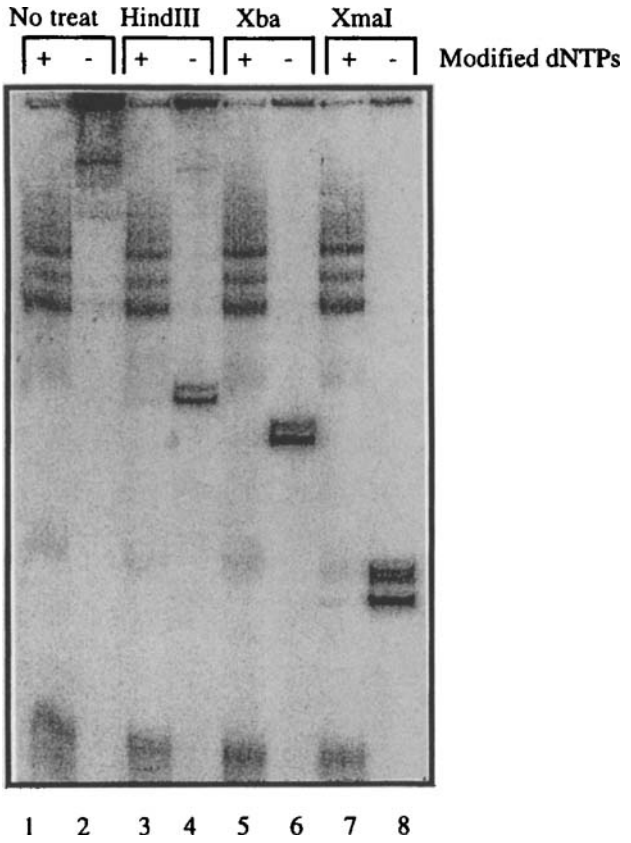

Figure 2: Restriction Enzyme digests of labeled (-) strand-M13 DNA. Lanes 1 and 2 are control lanes containing modified and unmodified DNA respectively. 3, 5, and 7 contain digests of modified DNA while Lanes 4,6, and 8 contain digests of the unmodified DNA.

modified heteroduplex DNA. In each case, the modified strand is labeled and the unmodified M13 template DNA is unlabeled. The fact that virtually no cleavage of the modified strand is observed with HindIII and XbaI might be expected since both sites contain the two modified bases within the recognition sequence. Even though some cleavage is seen at the XmaI site (comprised entirely of Cs and Gs), it too resists cleavage. This may be explained by the fact that in this case, the site is proximally flanked by an $A^{im}U^{aa}$ and a $U^{aa}A^{im}$ which might either inhibit enzyme binding and/or distort the recognition sequence. Such distortions of B-DNA are known to be induced when one strand contains positively charged nucleosides.[34] This indicates that these modifications would potentially cause single-stranded structures to be nuclease resistant as well as rigidify them via favorable charge-charge interactions.

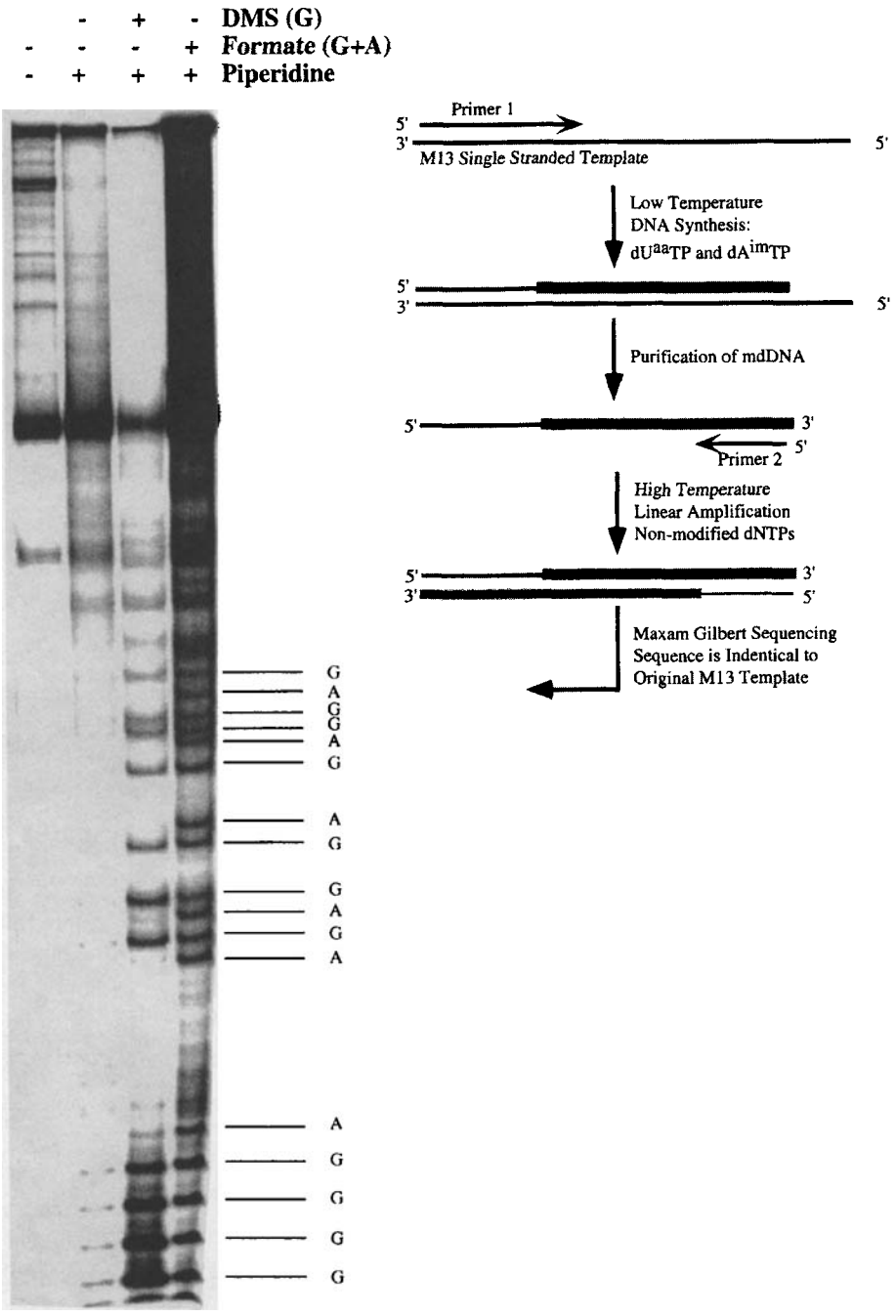

Figure 3: Maxam-Gilbert sequencing of recopied mdDNA. A naturally arrested band of mdDNA, indicated in Figure 1, was eluted and subjected to one-sided recopying at high temperature with Oligo#2. Lane 1: full-length linear product, Lane 2: full length product with piperidine treatment, Lane 3: DMS (G) reaction, Lane 4: Pip-formate (G+A) reaction. Schematic at right indicates the logic of the sequence analysis. Sequence in lanes 3 and 4 conforms to that of the M13 template.

Alternatively, one could also explain these results simply by misincorporation. However misincorporation would have to be *so* prevalent (i.e. that Im-dATP and/or AA-dUTP could replace G's and C's at the *XmaI* site), such that virtually 100% of the modified strands contain at least one misincorporation within the *XmaI* site. This translates into a 16% misincorporation rate per site - a phenomenon that would certainly have been detected by the sequencing in Figure 3 but is not.

We recognize that Figures 1 and 2 demonstrate that both $dU^{aa}TP$ and $dA^{im}TP$ are substrates for DNA polymerase, however the sequencing data in Figure 1 only prove that the unmodified dideoxynucleotide terminators are incorporated sequence specifically. There is no direct proof that *any* of the four bases preceding the terminator is incorporated sequence-specifically. Thus we wanted to address the issue of misincorporation. Because of the modifications, the mdDNA could not cleanly be sequenced by Maxam-Gilbert direct chemical sequencing. Consequently, the mdDNA was recopied into a complementary unmodified cDNA strand using Vent exo- DNA polymerase. This procedure not only permitted the sequencing of the mdDNA, but also demonstrated that the mdDNA could serve as a template for faithful recopying at high temperature, a requisite condition for eventual exponential amplification. To address this concern, one of the fortuitously arrested mdDNA products, estimated at approximately position +51, in Figure 1, was isolated. If faithfully synthesized, the mdDNA fragment would necessarily be complementary to the known sequence of its M13 template DNA. The sequence of Oligo #2 is then the would-be complement to the mdDNA fragment arrested at position +51. 5'-labeled Oligo #2 was used to recopy the mdDNA in a one-sided high temperature polymerization reaction in the presence of unmodified dNTPs and the extension products were then subjected to Maxam-Gilbert chemical sequencing. A one-sided linear amplification instead of a two-sided exponential amplification was deliberately chosen to avoid the possibility of amplifying contaminating amounts of unmodified M13 template DNA which would have given identical results even if the mdDNA was not a template for cDNA recopying. The use of a one-sided approach necessarily demands that the mdDNA, and only the mdDNA, serve as a template for cDNA synthesis. To be absolutely certain that no contaminating fragments of (+)-strand M13 DNA were present to give positive artifacts, a sham gel slice was excised from a position corresponding to the same position as where the mdDNA was isolated except that the sham lane was loaded with only M13 template

DNA. The contents were run as a negative control with Oligo #2. One does observe rather faint but detectable acid-labile purines in a region that should contain 6 pyrimidines (at the bottom of the gel, lane 4, Figure 3). These bands were also seen in the negative control (data not shown) and result from non-templated additions and/or primer-dimer effects, which compete with the one-sided extension. It is well known that sequence data close to the primer are often hard to interpret and are prone to artifacts. No such artifacts are seen higher in the gel: e.g. the second subsequence 5'AGG3' is recopied to give 5'CCT3' (Figure 3) with no observed acid-labile bands. We demonstrated a high degree of fidelity that the incorporation was faithful BOTH in the introduction of modified bases AND in the recopying by unmodified bases. Although we believe that the faint purine bands are entirely due to unrelated artifacts, the possibility exists for imperfect incorporation, especially when polymerases lack proofreading activity.

The logic behind this experiment is that both faithful incorporation of the modified dNTPs *and* faithful recopying of the mdDNA into non-modified cDNA are necessary to give a final product with the same sequence as the original M13 DNA template. The results of Figure 3 indicate that the initial incorporation as well as the subsequent read-out are faithful to the original sequence thus confirming that the fourth and final condition is satisfied for the use of both $dU^{aa}TP$ and $dA^{im}TP$ in a combinatorial in-vitro selection process.

Conclusions:

The results here demonstrate that two modified deoxyribonucleotide triphosphates are sequence-specifically incorporated to generate polymers bearing functionalities commonly found at the active sites of many hydrolytic enzymes where such groups play the roles of electrostatic stabilization, general acid-base catalysis, and metal ion coordination. The polymerized mdDNA can also be recopied into cDNA by a high temperature polymerase. Thus the use of $dA^{im}TP$ and $dU^{aa}TP$ in a combinatorial in-vitro selection scheme to select a metal-independent ribonuclease activity with a catalytic rate constant superior to that already observed in unmodified aptamers, would serve as a proof-of-concept.[35] However the role of the imidazole to chelate metals such as copper, zinc, cobalt, and calcium is not to be underestimated when seeking more active metal-dependent nucleases or for developing inhibitors of reverse transcriptase, anti-gene ligands, and activities such as esterase and aldolase.[36]

When a degenerate population of sequences must compete for polymerase activity, the question of uniform reactivity is generally pertinent to all combinatorial approaches. In selection schemes where the sequences must compete for the polymerases employed, sequences of a 'more average' composition might be more efficiently copied and thus would be expected to win out despite the fact that 'rare' sequences may be more active because of a certain structural motif (e.g.*i*-motif, G-quartet) due to a particular sequence composition. The concern for uniform reactivity may be exacerbated by the presence of modified dNTPs; multiple incorporations do indeed perturb the uniformity of dNTP incorporation. When a degenerate population of 10^{12} different sequences is examined, those sequences that contain fewer modifications may be polymerized and recopied more efficiently than those relatively rich in AT bearing the modifications. Thus, *a-priori*, selecting catalysts with such modifications would likely be disfavored except in the cases where the gain-of-function of incorporating dA^{im} and dU^{aa} offsets the polymerases' presumed preference for introducing nonmodified (Gs and Cs) and for recopying modification-poor strands. Although these results indicate that at least four dA^{im}'s can be sequentially introduced, whether more than 4-in-a-row can be incorporated remains unclear. Previously we demonstrated that the sequence $A^{im}U^{aa}A^{im}U^{aa}A^{im}$ could be readily polymerized on a complementary template[37]. Since only two imidazoles are probably sufficient for RNase activity, the concern for reduced sequence-space, may be exaggerated. Thus the use of a combinatorial selection scheme to achieve the proper orientation and scaffolding for only a few modified bases is of equal significance.

Depending on the nature and number of functional groups to be incorporated, the number of sequences amenable to polymerization and reamplification might be reduced. The difference between chemical and statistical diversity relates to the difference between antibodies and aptamers: One advantage of in-vitro selection of nucleic-acid based catalysts is that as many as 10^{15} different molecules can be sampled whereas peptide and antibody libraries are often limited to 10^{10} different molecules. However the chemical diversity in proteins probably more than compensates for reduced overall degeneracy of the population. This compensation is nicely illustrated by the fact that antibodies generated against a transition state analog: hexachloronorbornene-2,3-imide, catalyzed a Diels-Alder condensation reaction of tetrachlorothiophenedioxide and N-ethyl-maleimide whereas aptamers selected to bind to the same transition-state analog did not.[38]

In spite of the above comparison suggesting the superiority of antibody catalysts, selecting for activity rather than TSA biding, has resulted in nucleic-acid catalysts capable of stabilizing pentacoordinate phosphate for which no stable TSA exists. At least in this context, nucleic-acid catalysts may surpass antibodies. Similarly, they should, in theory, be able to stabilize other anionic species arising during the transition state of hydrolytic attack at carbon for which transition state analogs do exist. Having selected aptamers that bound to dyestuffs, Szostak and Ellington logically suggested the possibility of generating catalytic RNA molecules that would bind to transition state analogs in a similar fashion to catalytic antibodies.[39] However, most antibody-catalyzed hydrolytic reactions proceed via a negatively charged tetrahedral intermediate resembling phosphonates, phosphodiesters, and phosphoramidates which antibodies recognize in part by lysines, arginines, and histidines.[40] Significant rate acceleration via transition state stabilization is achieved because the antibody affinity for a TSA is often nanomolar.[41] To date, virtually no aptamer has been selected to bind a small, negatively charged molecule with nanomolar affinity.

Just how aptamers recognize small ligands has been a subject of active research. The solution and crystal structures, corroborating the affinity-elution data, showed that the aptamer-bound ATP (Kd = 1μM) was being recognized everywhere but on phosphate.[42-44] It is thus difficult to envision how an aptamer, totally devoid of a positively charged residue and replete with a negatively charged backbone could ever recognize a negatively charged TSA with high affinity at physiological pH.

It is certainly valid to try to understand the limitations of catalytic nucleic acids in the absence of groups playing the role of a positive charge and an imidazole. But it is increasingly challenging to imagine how aptamers will ever rival the catalytic potential of abzymes and moreover enzymes as might have been previously suggested.[45] In view of what now appear to be inherently and insurmountably low catalytic rate constants, several authors suggested that boosting catalytic potential by incorporating bases appended with a histidine or lysine residue would be "cheating".[46] However, primitive RNA catalysts may have very well cheated. Uracil, under primitive-earth conditions, can acquire several functionalities at the 5 position including an ammonium and an imidazole.[47] Such a catalyst presenting these two functionalities, so characteristic of protein active sites, would most certainly be interesting. This work indicates that such functionalities can be incorporated to expand and enhance the catalytic potential of in-vitro selected nucleic acid catalysts. Selections for both metal dependent and

independent RNase activities are underway to examine whether the limitations on sequence space will be offset by a gain of function due to incorporation of modified bases.

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