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A Specific, Highly Active Malate Dehydrogenase by Redesign of a Lactate Dehydrogenase Framework

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Three variations to the structure of the nicotinamide adenine dinucleotide (NAD)–dependent L-lactate dehydrogenase from *Bacillus stearothermophilus* were made to try to change the substrate specificity from lactate to malate: $Asp^{197} \rightarrow Asn$, $Thr^{246} \rightarrow Gly$, and $Gln^{102} \rightarrow Arg$). Each modification shifts the specificity from lactate to malate, although only the last ($Gln^{102} \rightarrow Arg$) provides an effective and highly specific catalyst for the new substrate. This synthetic enzyme has a ratio of catalytic rate (k_{cat}) to Michaelis constant (K_m) for oxaloacetate of $4.2 \times 10^6 M^{-1}\ s^{-1}$, equal to that of native lactate dehydrogenase for its natural substrate, pyruvate, and a maximum velocity (250 s^{-1}), which is double that reported for a natural malate dehydrogenase from *B. stearothermophilus*.

LACTATE DEHYDROGENASE (LDH) and malate dehydrogenase (MDH) are structurally related enzymes (*1–5*) that use the same coenzyme to bring about the redox interconversion of keto- and hydroxy acids by kinetically comparable mechanisms (*6–8*) as shown in Scheme I.

Yet each enzyme is highly selective for its substrate (NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced NAD). For example, the MDH from *Thermus flavus* has a k_{cat}/K_m value for oxaloacetate of $6.7 \times 10^6 M^{-1}\ s^{-1}$ and one for pyruvate of $7.2 M^{-1}\ s^{-1}$ (*9*). The LDH from *Bacillus stearothermophilus* has k_{cat}/K_m values for pyruvate and oxaloacetate of 4.2×10^6 and $4 \times 10^3 M^{-1}\ s^{-1}$, respectively.

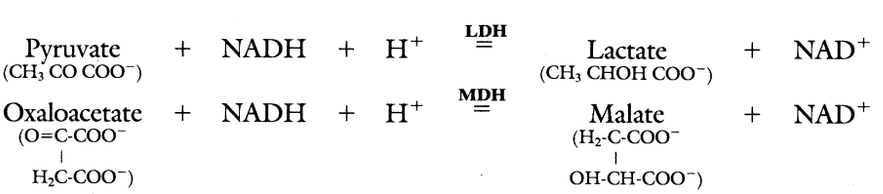
In searching for the basis of this sharp discrimination between substrates there are two sources of reference. The first is comparison of gene-derived and protein-derived amino acid sequences: the primary structures of 20 LDHs and 6 MDHs are known. The second is crystallographic: there are several x-ray structures of LDH in its apo (free enzyme) (*10*), binary (enzyme-NADH) (*11*), and ternary (enzyme-NADH-substrate analog) (*12–14*) forms and a binary structure of MDH (*2, 15*).

Despite these guidelines, it was not possible to predict with conviction the elements of the proteins that select one substrate and reject the other. For example, there is no MDH structure with a bound substrate analog. A comparison of the amino acid sequences of LDH and MDH is not definitive. Despite their similarity in secondary and tertiary structure demonstrated by crystallographic studies, they show only a limited sequence identity; 19% between the LDH from *B. stearothermophilus* (*16*) and the MDH from *T. flavus* (*17*), both thermophilic prokaryotes. Even in the sections of polypeptide that we judge constitute the catalytic pocket, the identity is only 35% and any or all of the 31 amino acid changes around the active site could contribute to the difference in specificity (*18*).

```
     * *  * *  *      **        **  *   * *
MDH GAAPRKAGMER   VGNP   MT-RLDHNR   GNHSS
LDH G-ANQKPGETR   ATNP   SGTILDTAR   GEHGD
    98  -  109  138-141  163  -  171  193-197
    (no 104)
                        **  **       *
                   AQ-RGAAIIQARGAS   SAAN
                   VRDAAYQIIEKKGAT   GIAM
                   232     -   246  249-252
```

One method of testing hypotheses for substrate discrimination is to use mutagenesis to synthesize the putative new dehydrogenase structure and test its substrate specificity. This approach is not dissimilar to earlier chemical tests of catalytic mechanisms by the synthesis of a thiol proteinase from a serine proteinase (*19*). We report here successful attempts to change the specificity of *B. stearothermophilus* LDH to discriminate against pyruvate and in favor of oxaloacetate. The designs were based on a detailed understanding of the mechanism of LDH, an understanding acquired by a combination of chemical modifications, site-specific amino acid substitutions, transient and steady-state kinetic measurements, and the exploitation of optical probes (*3, 7, 20–24*). We expect the same catalytic mechanism (*6*) will prevail in the redox interconversion of oxaloacetate and malate and so make no alteration to the residues that have identifiable catalytic roles.

The spatial relation of residue 197 to the active site is shown in Fig. 1, the negative charge of the native aspartate carboxylate group is 0.8 nm from the substrate. This aspartate residue, unlike Asp^{168} (*22*), has no known catalytic function, but is always a neutral residue in MDH. If charge conservation (*24*) plays a large part in substrate discrimination, then substituting this residue for the neutral asparagine ought to select against pyruvate (charge −1) and in favor of oxaloacetate (charge −2). The steady-state catalytic properties of this modified enzyme (Table 1) broadly fulfill the prediction. There is a 32-fold reduction in catalytic efficiency against pyruvate (as measured by k_{cat}/K_m) and only a 1.3-fold reduction against oxaloacetate; the selectivity, therefore, shifts toward oxaloacetate (Fig.



Scheme I

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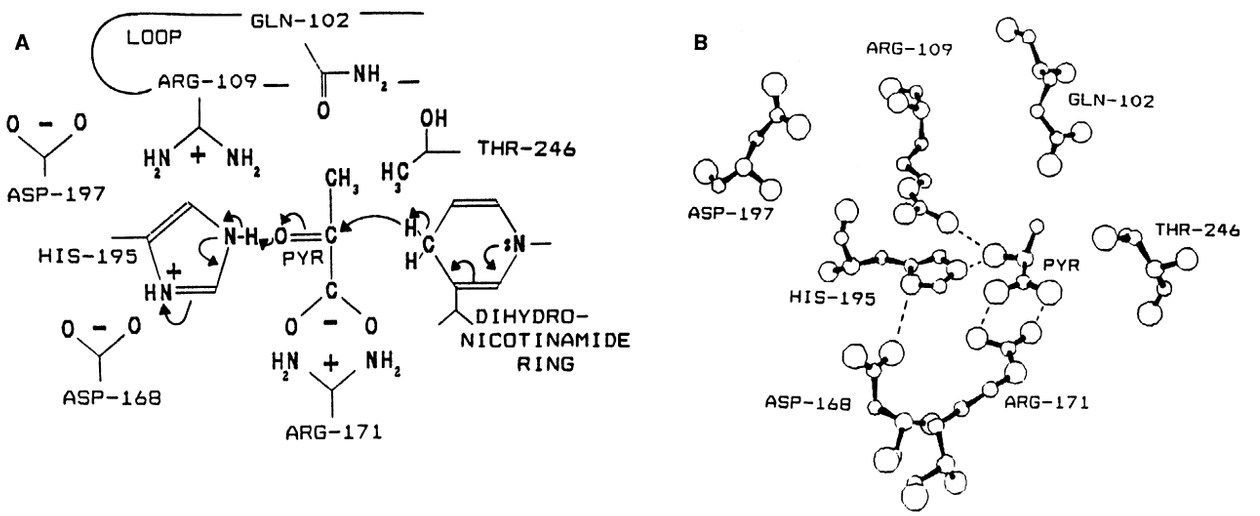

Fig. 1. The active site environment and catalytic mechanism of native lactate dehydrogenase. (**A**) The chemical environment of pyruvate in the native enzyme–NADH–substrate complex. The residues responsible for catalytic efficiency are shown; for example, Arg^{109} polarizes the carbonyl bond (*20*), His^{195} donates a proton (*3*), Arg^{171} binds and orients the substrate (*21*), and Asp^{168} stabilizes the protonated His^{195} (*22*). Residues being tested for substrate selection are Gln^{102}, Asp^{197}, and Thr^{246}. The electron pair movement during pyruvate reduction is indicated and results in the donation of a proton from His^{195} to the substrate carbonyl oxygen and a hydride ion from the dihydronicotinamide ring to the carbonyl carbon. (**B**) The geometric arrangement of the active site is from the coordinates of the M4-isoenzyme of pig LDH–NADH–oxamate complex (*11*). For clarity, the coenzyme dihydronicotinamide ring is omitted, but otherwise the same structures are included as in (A).

2). This shift is largely produced by the 10-fold lowering of the K_m for oxaloacetate and an 11-fold increase in K_m for pyruvate. This result emphasizes the influence of overall charge balance on the stability of ternary complexes in this enzyme, but the effect of the substitution is insufficient to reverse the enzyme's selectivity and produce an effective MDH.

Inspection of the model structure of the LDH ternary complex suggests that substrates with larger side chains than pyruvate would extend into the region occupied by Gln^{102}, Arg^{109}, and Thr^{246} (Fig. 3, also Fig. 1B). To accommodate the increased size of oxaloacetate, we chose to enlarge the volume of this binding pocket. Of the available options, Arg^{109} is involved in catalysis and enhances the hydride transfer rate by at least three orders of magnitude (*20*), so its substitution would cripple catalysis, and residue 102 is always larger in MDH than in LDH. Residue 246, threonine in all LDHs, is always smaller in MDH and provides a site to accommodate a larger substrate. This residue was replaced by glycine. Like the $Asp^{197} \rightarrow$ Asn mutant, this modified enzyme shows a shift in specificity away from pyruvate and toward oxaloacetate (Table 1), but more pronounced. The $Thr^{246} \rightarrow$ Gly mutant shows a preference for the new substrate over the old (Fig. 2) resulting from a 3200-fold reduction in the k_{cat}/K_m for pyruvate and a slight enhancement in the case of oxaloacetate. The improved K_m for the new substrate, now 0.2 m*M* rather than 1.5 m*M*, suggests that Thr^{246} in the LDH active site can select effectively against oxaloacetate binding.

The Arg^{171} to substrate carboxyl bond in LDH is 5 kcal mol^{-1} stronger than an ion pair with the amino group of lysine (*21, 25*). In native LDH, the binding of coenzyme and substrate induces a rearrangement of the protein structure in which a loop of polypeptide chain (residues 98 to 110), originally lying away from the body of the protein (*10*), closes over the active site (*12–14*). Computer graphic modeling of the ternary structure suggests that Gln^{102} on this loop, if changed to arginine, could form a good ion pair with the 3-carboxyl group of a model oxaloacetate substrate (Fig. 2). Residue 102 is variable in LDH sequences, but an argi-

Table 1. Kinetic properties of native and modified enzymes. Mutant enzymes were made by the oligonucleotide mismatch procedure of Winter *et al.* (*29*) carried out on the *lct* gene NCA 1503 (*16*) as we have described previously (*20*). The enzymes were produced from a high expression plasmid pKK223-3 in *Escherichia coli* (*16*), the wild type being isolated by oxamate-Sepharose affinity chromatography (*30*) and the mutants, which no longer bind well to the pyruvate analog, by triazine-Sepharose and ion-exchange chromatography (*21*). In all cases the entire gene was resequenced from the pKK223-3 plasmids isolated from the actual culture used for the large-scale protein preparation in order to ensure that only the directed mutations had arisen. The kinetic constants of k_{cat} and K_m were determined in steady-state experiments at 25°C in 100 m*M* triethanolamine HCl-NaOH buffer, *p*H 6.0 (*21, 22*), containing 5 m*M* fructose-1,6-diphosphate. The primary deuterium kinetic isotope effect (described as k_H/k_D in the table) is a ratio of the reaction rates at saturating coenzyme and substrate concentrations with NADH and 4-[^{2}H]NADD as reductants. (Values of k_{cat} are the means of four experiments. Individual measurements were all within ±5% of the quoted mean.) These rates were measured in single-turnover, stopped-flow experiments from the first-order decay in the concentration of reduced coenzyme (*20, 22*), the only exception is the ratio determined for the wild-type enzyme with oxaloactate. In this case, because of the potent effect of even a low level of pyruvate contamination from substrate decarboxylation, the ratio was measured from steady-state assays (the initial "burst" of contaminant pyruvate reduction was ignored).

Enzyme	Pyruvate				Oxaloacetate			
	k_{cat} (s^{-1})	K_m (m*M*)	k_{cat}/K_m (M^{-1} s^{-1})	k_H/k_D	k_{cat} (s^{-1})	K_m (m*M*)	k_{cat}/K_m (M^{-1} s^{-1})	k_H/k_D
Native	250	0.060	4.2×10^6	1.0	6.0	1.5	4.0×10^3	2.8
$Asp^{197} \rightarrow$ Asn	90	0.66	1.3×10^5	1.3	0.50	0.15	3.0×10^3	2.0
$Thr^{246} \rightarrow$ Gly	16.0	13.0	1.3×10^3	2.8	0.94	0.20	4.7×10^3	2.1
$Gln^{102} \rightarrow$ Arg	0.9	1.8	5.0×10^2	2.6	250	0.06	4.2×10^6	1.0

nine in known MDHs.

The effect of this mutation is very large: the k_{cat}/K_m for oxaloacetate is improved by three orders of magnitude while that for pyruvate is worsened by four orders of magnitude (Table 1). The new enzyme is not only 8400 times as effective against oxaloacetate as against pyruvate (Fig. 2) but catalyzes oxaloacetate reduction with a k_{cat}/K_m of $4.2 \times 10^6 M^{-1}$ s^{-1}. This value, and the k_{cat} (250 s^{-1}) are as good as those achieved by LDH with its natural substrate.

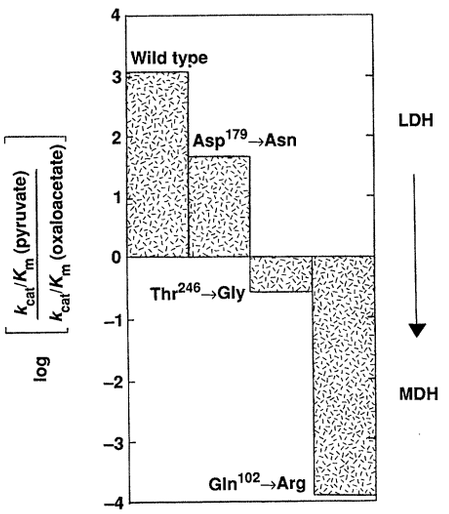

Fig. 2. Shift in substrate specificity of the redesigned enzymes. The values for catalytic efficiency (k_{cat}/K_m) shown in Table 1 are here expressed as the logarithm of the ratio of pyruvate to oxaloacetate for the wild-type and mutant enzymes. The histogram emphasizes the extent to which each mutation shifts the enzymes' selectivity toward oxaloacetate to create an effective MDH.

The maximum rate of catalysis in native LDH with pyruvate as the substrate at 25°C is not the rate of transfer of the hydride ion from coenzyme to the substrate-carbonyl group. There is no primary kinetic deuterium isotope effect on k_{cat} (*20*). The upper rate for the reaction is limited by a structural rearrangement of the enzyme-NADH-substrate complex prior to the chemical step (*3*). Our recent experiments with a genetically inserted single tryptophan fluorescence probe (*23*) show that this rearrangement is dominated by the closure of the active-site loop (residues 98 to 110) from an open structure, assumed to be like that in crystals of dogfish apo-LDH (*10*), to a closed structure that varies little from species to species or ternary complex to ternary complex (*12–14*).

In contrast, all but one of the mutant catalytic reactions measured in our study show primary kinetic isotope effects with a deuterated coenzyme. That is, the rate of the chemical step becomes slow in comparison to the rate of the loop closure because the protein environment is less effective in promoting hydride transfer. The exception is the $Gln^{102} \rightarrow$ Arg enzyme with oxaloacetate as substrate. Here the maximal rate of 250 s^{-1} is identical to native enzyme with pyruvate and, likewise, shows no primary kinetic isotope effect. We infer that this is an "intrinsic" loop-closure rate and will set the upper limit of reaction velocity in this enzymatic framework (unless the hinge regions of the mobile loop are redesigned).

Redesigning the LDH enzyme framework to accept oxaloacetate as a substrate poses the problem of introducing a new negatively charged carboxyl group into an active site that is isolated from solvent water in the catalytically active ternary complex. When the mobile surface loop (98 to 110) closes over the bound substrate and coenzyme, water is excluded from the reaction center and Arg^{109} moves 0.9 nm from a position in the solvent to one in which it makes a close hydrogen-bonding contact with the substrate carbonyl group. In this arrangement, the carbonyl bond is charge-polarized (*19*), and a hydride ion from the coenzyme can attack the positively charged carbonyl carbon while a proton from His^{195} is transferred to the negatively charged oxygen (Fig. 1a). The efficiency of this mechanism is reduced 1000-fold (k_{cat}/K_m) when the substrate methyl group is replaced by a carboxymethyl. In this case, in order to achieve a closed reaction center, the extra carboxyl group must be "dissolved" by the protein side chains of the catalytic pocket without any compensatory positive charge. The energetic cost of this is a loss of 4 kcal in the stability of the enzyme transition state complex formed with the native enzyme and oxaloacetate as substrate.

Conversely, in the $Gln^{102} \rightarrow$ Arg enzyme with pyruvate as substrate, the catalytically active complex must form with a buried and unpaired guanidinium cation in the active site. Here there is a loss of 5.5 kcal in the stability of the enzyme transition-state structure. It is only when this modified enzyme binds oxaloacetate that the charges can pair to desolvate the groups and accommodate them in the protein interior (Fig. 3). The energy of solvation of arginine residues is particularly large (*26*). In this arrangement a catalytic activity similar to that of the wild-type enzyme with the natural substrate is restored.

These experiments show how an induced protein rearrangement is harnessed to provide extreme discrimination between two very similar substrates. The induced fit brings not only the correct substrate recognition groups into the active center, but that charge-pairing also enables very important catalytic but charged residues (Arg^{109},

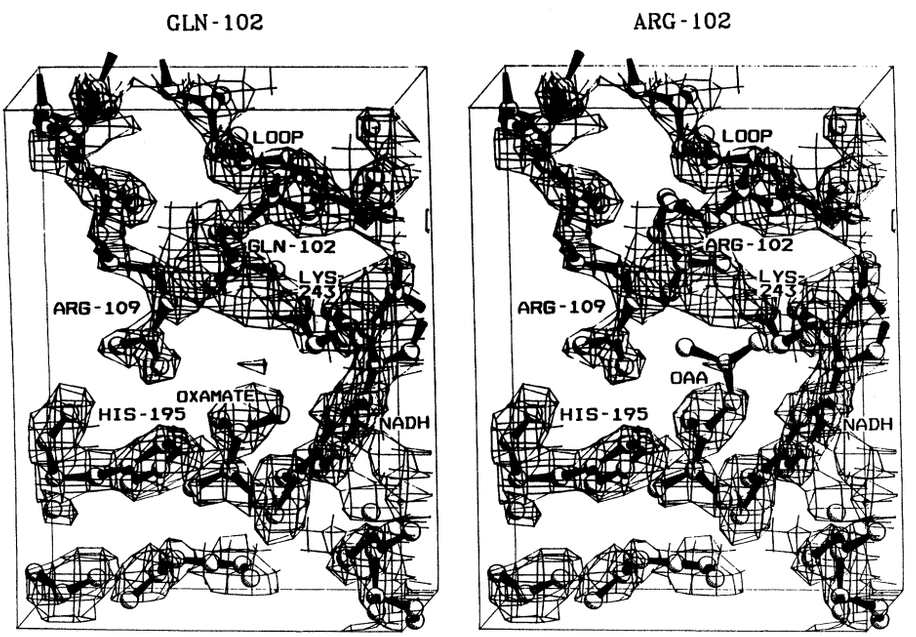

Fig. 3. Model of the $Gln^{102} \rightarrow$ Arg enzyme active site with bound oxaloacetate. The original design was based on models of *B. stearothermophilus* LDH active center built by replacing the side chains of two LDH structures (*31*) with the side chains from the gene-derived amino acid sequence of the bacterial enzyme (*16*). Since that time we have determined and partly refined a pig M4 LDH–NADH–oxamate structure to 0.2 nm resolution [at present the R-factor is 33% for 37,000 reflections (*11*)]. The electron density in this more accurate structure is used to demonstrate (**left**) the space between the NH_2-group of bound oxamate (and the presumed CH_3-group of active pyruvate) and the amide group of Gln^{102} and (**right**) a model in which that space is occupied by the extra carboxyl group of oxaloacetate and the extended side chain when the glutamine is replaced by Arg^{102}.

Asp[168]) to rearrange to their catalytically effective positions. The 8400-fold discrimination between a methyl and a carboxymethyl side chain is only achieved in an environment in which the substrate is completely solvated by protein groups. Lack of detailed understanding of protein intramolecular mobility may account for other attempts (*27*) to alter enzyme-substrate specificity being less than entirely successful.

Our results show it is possible to design and make a new MDH, which is by any standards a good enzyme and about twice as active as that which is naturally evolved in the organism from which the gene was derived (*28*). In the case of well-studied protein frameworks where new enzymes can be designed and made by small changes in residues close to the protein surface, we may hope it will soon be less laborious to redesign an existing and thermally stable enzyme framework for a new target substrate than to search for a new enzyme activity from natural organisms.

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18. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Translation of Unspliced Transcripts After Heat Shock

H. JOSEPH YOST* AND SUSAN LINDQUIST

Severe heat shocks block the splicing of intervening sequences from messenger RNA precursors. The RNA's that accumulate after a severe heat shock have normal transcription start sites and are uncut at both their 5′ and 3′ splice junctions. Some of these unspliced transcripts leave the nucleus and enter the pool of cytoplasmic messenger RNA. Translation of these RNA's proceeds into their intervening sequences, resulting in the production of abnormal proteins. Thus, the repression of normal transcription, which usually accompanies the heat shock response, may protect the cell from the large-scale synthesis of abnormal RNA's and aberrant proteins.

ALL CELLS RESPOND TO HIGH TEMperatures by inducing the synthesis of heat shock proteins (hsp's) and at least partially suppressing the synthesis of normal cellular proteins (*1*). In *Drosophila melanogaster* cells, transcription of heat shock genes is rapidly induced by a shift from the normal growing temperature of 25°C to temperatures between 29° and 39°C. Heat-shock transcription is maximal at 37°C; at this temperature the transcription of previously active genes is repressed (*2*). Heat shock also elicits a complete change in the specificity of translation. While heat-shock mRNA's are translated with very high efficiencies at 37°C, preexisting mRNA's are translationally inactive. These messages are not degraded, but are quantitatively retained and translated during recovery from heat shock (*3*).

We have shown that another basic aspect of gene expression in *Drosophila* cells is altered by heat shock; the splicing of intervening sequences from mRNA precursors is blocked at high temperatures and remains blocked during the initial stages of recovery at normal temperatures. This block in splicing was first discovered because it greatly reduces the synthesis of the 83-kD heat shock protein (hsp83). The *hsp83* gene is the only hsp gene in *Drosophila* that contains an intervening sequence, and its protein is therefore the only hsp so effected. However, the block in splicing is not specific to *hsp83* transcripts. When the intron-containing alcohol dehydrogenase gene is placed under the control of a heat-inducible promoter, its transcripts are also not spliced at high temperatures (*4*).

As is the case with other toxic effects of heat, the hsp's appear to mitigate the effects of heat on RNA splicing. When *Drosophila* cells are given a mild heat treatment, which induces the synthesis of hsp's, before they are exposed to more severe temperatures, RNA splicing is protected. The protective effect of the prior treatment is blocked when cycloheximide is added before, but not after, the synthesis of hsp's (*4*). The disruptive effects of severe heat shocks on RNA splicing are highly conserved and are found in yeast (*5*), trypanosome (*6*), plant (*7*), and mammalian cells (*8*). The protective effects of mild heat treatment have been demonstrated for both *Drosophila* and yeast cells (*4, 5*).

In all eukaryotes the excision of intervening sequences to form mature messenger RNA's begins with the incorporation of RNA polymerase II transcripts into spliceosome complexes consisting of small nuclear (snRNP) and heteronuclear (hnRNP) ribonuclear protein particles. Two catalytic reactions then occur. First, the precursor RNA is cleaved at the 5′ exon-intron junction with the concurrent formation of a lariat structure, in which the 5′ guanosine of

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