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REVIEW

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Initiation of Transcription – a General Tool for Affinity Labeling of RNA Polymerases by Autocatalysis

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It is well known that the transcription of structural genes in eukaryotic cells is a highly regulated process. For this regulation, the DNA template contains numerous specific sequences which control the frequency and the precise start of initiation of transcription. There is ample evidence that these regulatory nucleotide sequences are recognized by a multitude of protein factors. In the presence of these factors, specific transcripts can be synthesized in vitro from suitable templates with highly purified RNA polymerase B (II)[1]. In the absence of these factors very few free RNA chains of defined length are made. One possible explanation for this observation is that purified eukaryotic RNA polymerase B (II) – as opposed to the eubacterial enzyme – does not bind to specific DNA sequences directly. Instead, it is believed that the enzyme recognizes a specific complex of factors with their cognate promotor elements for initiation of transcription[2].

Start of RNA synthesis in vitro

We were quite surprised when we found exceptions to the aforementioned rule. In the following experiment, we used as template a blunt-ended DNA fragment of 1303 bp length (Fig. 1) which contains the promoter region and RNA initiation site followed by 128 bp of the coding region of one of the zein genes in maize[3]. Upon incubation with highly purified RNA polymerase B (II) from wheat germ[4] together with radioactively labeled substrate, RNA is formed which migrates during electrophoresis in the form of two closely spaced, discrete bands with apparent lengths of 750 and 650 nucleotides (Fig. 1a).

Enzymes:
DNA-directed RNA polymerase, nucleoside-triphosphate:RNA nucleotidyltransferase (DNA-directed) (EC 2.7.7.6);
DNA-directed DNA polymerase, deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase (DNA-directed) (EC 2.7.7.7);
alkaline phosphatase, orthophosphoric-monoester phosphohydrolase (alkaline optimum) (EC 3.1.3.1);
nucleotide pyrophosphatase, dinucleotide nucleotidohydrolase (EC 3.6.1.9);
deoxyribonuclease I, (EC 3.1.21.1);
ribonuclease T_2 (EC 3.1.27.1);
ribonuclease (pancreatic) (EC 3.1.27.5);
Tritirachium alkaline proteinase (EC 3.4.21.14), also named proteinase K.

Abbreviation:
bp, base pairs.

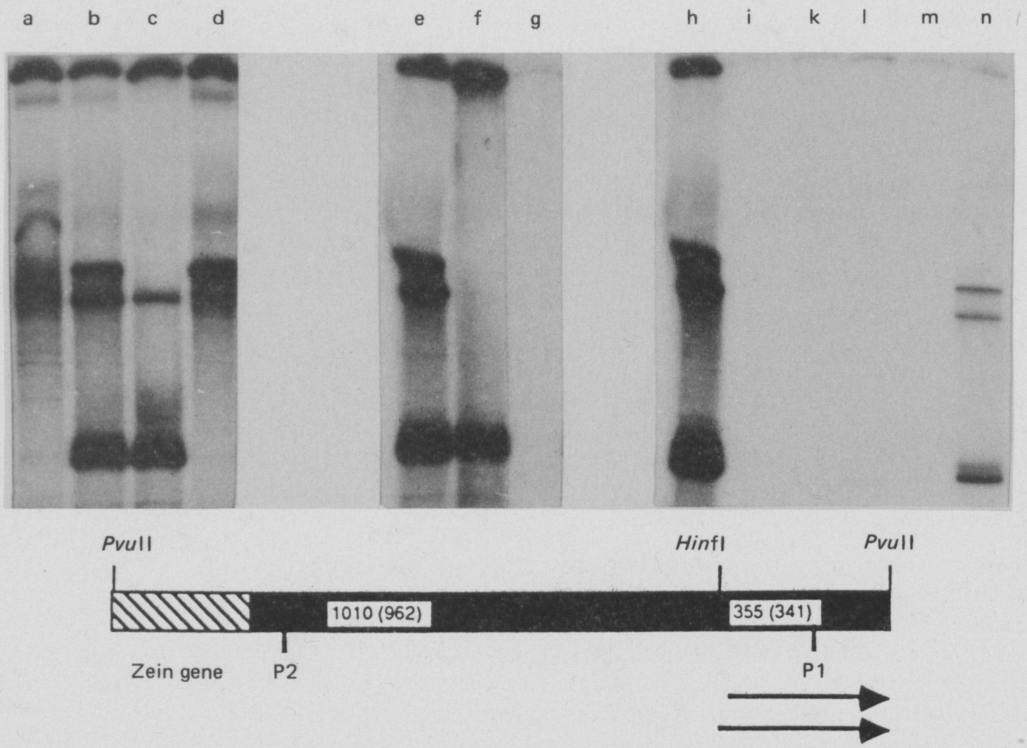

Fig. 1. Influence of specific cleavage of a promoter-containing template on transcription by highly purified RNA polymerase B (II) from wheat germ.

Lane a: 0.25 pmol of a 1303 bp DNA fragment (shown schematically) which was obtained by digestion of a pUC12 vector containing a zein gene[3] with *Pvu*II, was incubated with all four ribonucleoside triphosphates and 0.18 μg enzyme[4] for 30 min at 30 °C under conditions similar as described[7], b: as in a, but template cleaved with *Hin*fI; c: as in a, but with the 341 bp *Hin*fI fragment as template; d: as in a, but with the 962 bp *Hin*fI fragment; e: as in b; f: as in e, but followed by incubation with 2 μg deoxyribonuclease I; g: as in e, but followed by incubation with 2 μg ribonuclease A; h: as in e; i: as in h, but without template; k: as in h, but without enzyme; l: as in h, but without $MnCl_2$; m: as in h, but in presence of 1 μg/ml α-amanitin; n: as in h, but in presence of 2 μg/ml heparin. Autoradiographic analysis was performed after electrophoresis in a 4% polyacrylamide gel containing 6M urea.

However, when the template is first cleaved at its single *Hin*fI site two additional very intense radioactive bands with length of approximately 330 and 340 nucleotides appear (Fig. 1b, e, h). All four labeled bands consist of RNA since they can be hydrolysed with ribonuclease (Fig. 1g). However, the radioactivity of the upper two bands, consists of labeled ribonucleotides covalently bound to DNA since the radioactivity migrates out of the gel upon incubation with deoxyribonuclease (Fig. 1f). Obviously, without the specific cleavage of this template, RNA polymerase B (II) does not initiate de novo but only elongates at nicks or ends of the template. This is in agreement with earlier observations[5]. All four radioactive bands are not formed in the absence of enzyme (Fig. 1k) nor in the absence of DNA (Fig. 1i) or $Mn^{2\oplus}$ (Fig. 1l) or in the presence of either 1 μg/ml α-amanitin (Fig. 1m) or 2 μg/ml heparin (Fig. 1n). The bands are synthesized when 0.8mM $Mn^{2\oplus}$ is replaced by 8mM $Mg^{2\oplus}$ (data not shown). The two RNA bands initiated de novo are transcribed from the same *Hin*fI cleavage fragment of approximately 340 bp in length as evident from experiments where the separated *Hin*fI fragments were used as template (Fig. 1c, d). The sticky ends of the *Hin*fI cleavage site, present on both fragments, are alone not sufficient to induce the de novo initiation of RNA synthesis. This conclusion is supported by experiments with an isolated DNA fragment 316 bp long obtained by cleavage of pUC12 with *Pvu*II and with another fragment of 521 bp obtained by cleavage of pUC12 with *Alu*I. Only with the 316 bp fragment are large amounts of free RNA synthesized after cleavage of the template with *Hin*fI (data not shown) (H. Mosig, Ph.D. thesis, Fakultät für Chemie und Pharmazie der Ludwig-Maximilians-Universität München, in preparation). Probably some additional sequence requirements, missing in the latter fragment, must be met. However, the protruding 5′-end of the template is essential for the de novo synthesis. After extension of the

recessive 3′-ends with DNA polymerase I, the de novo RNA product is no longer formed (Fig. 2a). Both newly initated RNA chains start with ATP since they become labeled only when [γ-^{32}P]ATP is used as substrate (Fig. 2b). To determine the start site, the 5′-terminal 35 nucleotides of the RNA of 330 nucleotides length were sequenced.

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DNA  5'-AATCAAAATAGATGTATACCTAACATCAGCAAATGGAAATAAAA... -3'
     3'-  GTTTTATCTACATATGGATTGTAGTCGTTTACCTTTATTTT... -5'

RNA          pppAGAUGUAUACCUAACAUCAGCAAAUGGAAAUAAAA...
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Comparison of this sequence with that of the template[3] reveals that the start site of RNA synthesis is at the 7th base pair downstream of the double stranded part of the *Hin*fI cleavage site. This start is not the one selected in vivo and in vitro RNA synthesis proceeds opposite to the in vivo direction (scheme in Fig. 1).

Abortive initiation

The start of the RNA polymerase reaction in vitro can also be studied by abortive initiation[6,7]. Here, RNA polymerase is incubated with template and an incomplete set of ribonucleoside triphosphates as substrate. Under these conditions mainly di- or trinucleotides are formed which are readily released from the transcription complex.

$$pppN_1 + pppN_2 \rightarrow pppN_1pN_2 + pp$$
$$pppN_1pN_2 + pppN_3 \rightarrow pppN_1pN_2pN_3 + pp$$

The product contains at its 5′-terminus the intact triphosphate group of the starting nucleotide. Under abortive initiation conditions the first step of the RNA polymerase reaction is continuously repeated. This reaction is catalysed by eubacterial RNA polymerases[6] as well as by eukaryotic RNA polymerase B (II)[7,8]. In the case of the highly purified eukaryotic enzyme, however, it is not known if abortive initiation requires the same conditions as the initiation of unprimed synthesis of RNA. In vivo the start site of eukaryotic mRNA synthesis is much less precisely defined than in bacteria and may start at several nucleotides within a small sequence[9]. Therefore a direct comparison of the sequence of the product of abortive initiation with the 5′-terminus of the RNA formed in vivo is inconclusive. If under our experimental conditions (Fig. 1) abortive initiation really represents the initial step of de novo RNA synthesis in vitro, one would expect to observe significant dinucleoside tetraphosphate synthesis only after cleavage of the template with *Hin*fI and with the shorter of the two *Hin*fI cleavage fragments. Indeed, only the shorter fragment leads to the formation of large amounts of pppApU (Fig. 3B). To a small extent pppApA can be synthesized on both fragments (Fig. 3A, E). If abortive initiation occurs exactly at the same site of the template as the synthesis of RNA with the sequence pppApGpA at its 5′-terminus starts, then formation of pppApG would be expected, but is not observed (Fig. 3C, G). Also a combination of ATP and CTP is inactive (Fig. 3D, H). In agreement with earlier studies[7] these observations indicate that pppApU is the preferred product if allowed by the sequence of the template. A distinct stimulation of abortive initiation, similar to the increase in synthesis of free RNA when the template is cleaved with *Hin*fI, is also observed with the DNA fragment of 316 bp length obtained by cleavage of pUC12 with *Pvu*II (data not shown).

It was shown above that the protruding 5′-terminus of the *Hin*fI cleavage site is necessary for the induction of de novo RNA synthesis in vitro (Fig. 2a). The same is found for pppApU synthesis. Repairing the gap of the *Hin*fI cleavage

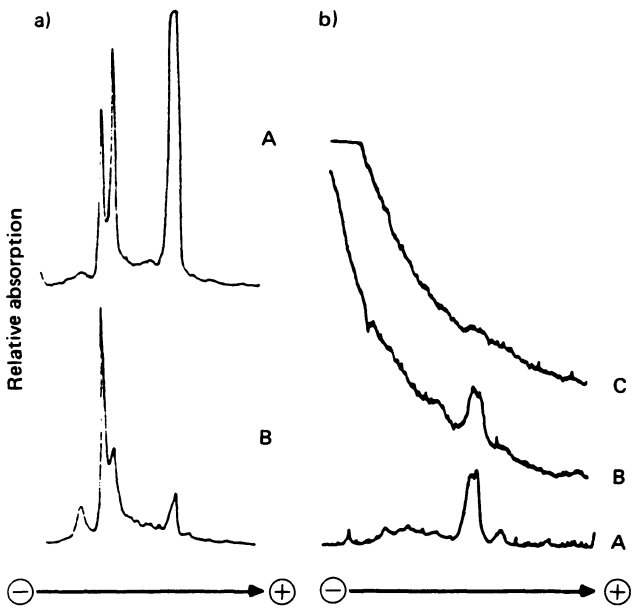


Fig. 2. Influence of the protruding ends of the template on transcription and determination of the 5′-nucleotide of the transcript (densitometric tracing of autoradiography).

a) Experiment A: transcription of the *Hin*fI-cleaved template was done as described in Fig. 1, lane b; experiment B: the protruding ends of the *Hin*fI-cleaved fragments were filled by incubation with DNA polymerase I (Klenow fragment) and the template was reisolated by phenol treatment and transcribed as before. b) Tracing A: transcription in the presence of [α-^{32}P]UTP; tracing B: in the presence of [γ-^{32}P]ATP; tracing C: in the prescence of [γ-^{32}P]GTP as labeled substrate.

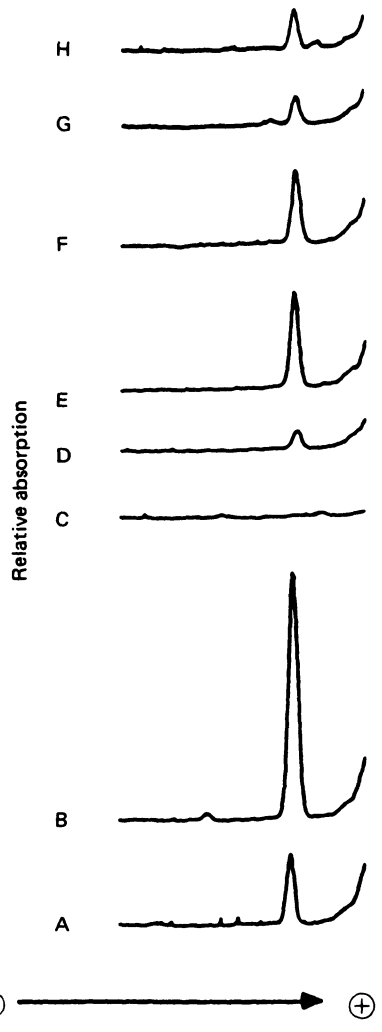


Fig. 3. Influence of specific cleavage of a promoter-containing template on the abortive initiation by wheat germ RNA polymerase B (II).

The experiments were carried out under identical conditions as used for transcription (Fig. 1) except that only one or two substrates were added. The dinucleoside tetraphosphates were separated by electrophoresis in a 25% polyacrylamide gel containing 6M urea. The densitometric tracings of the autoradiography are shown. Template: the 341 bp (A–D) or 962 bp (E–H) fragment obtained by *Hin*fI cleavage of the 1303 bp fragment described in Fig. 1. Substrate: [α-^{32}P]ATP (A, E); [α-^{32}P]ATP + UTP (B, G); [α-^{32}P]ATP + GTP (C, G); [α-^{32}P]ATP + CTP (D, H).

site leads to the loss of template activity also in abortive initiation (Fig. 4).

The following conclusions may be drawn from these experiments:

1) In the absence of additional protein factors, eukaryotic RNA polymerase B (II) from plants can initiate selectively the synthesis of free RNA of defined length. This is in agreement with observations made with RNA polymerase B (II) from calf thymus[10].

2) Efficient abortive initiation by purified eukaryotic RNA polymerase B (II) requires properties of the template similar to those required for the unprimed synthesis of RNA of specific length.

Labeling of the active site by autocatalysis

The findings described above suggest an application of an elegant method developed for labeling the region which contains the active center of *Escherichia coli* RNA polymerase[11,12] to eukaryotic RNA polymerases. The essential features of this method, as it is used in most of our experiments, consists of incubating the RNA polymerase with a chemically reactive nucleotide derivative in the absence of template and substrate[13]. Numerous different derivatives have been synthesized[14,15]. The chemical structure of only one of them is shown.

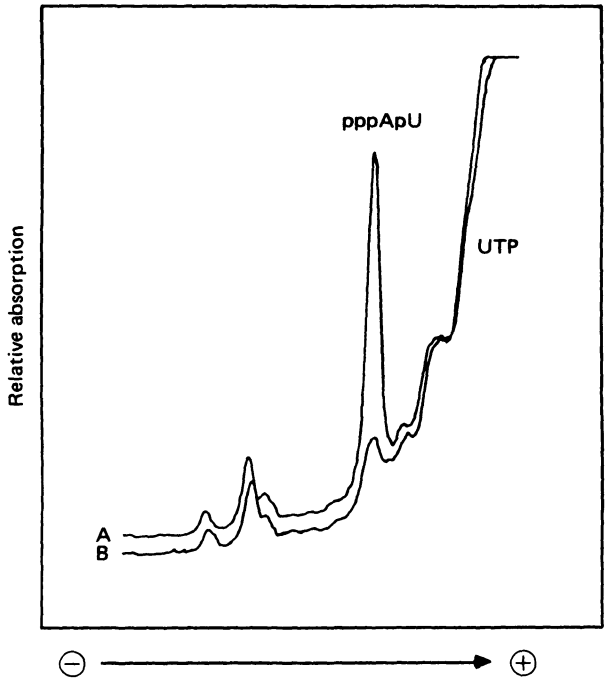
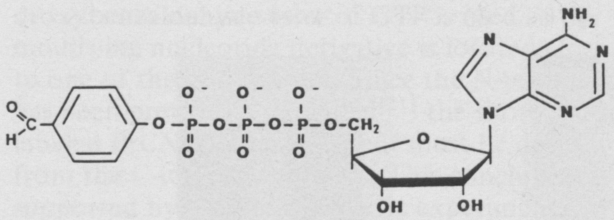


Fig. 4. Influence of the protruding ends of the template on abortive initiation.

The densitometric tracings of the autoradiography of the electrophoretic separation are shown. Upper tracing: abortive initiation with the *Hin*fI-cleaved template (Fig. 1) with ATP and [α-^{32}P]UTP as substrate as described in Fig. 3. Lower tracing: The same experiment with the *Hin*fI-cleaved template whose protruding ends had been filled by incubation with DNA polymerase I (Klenow fragment).



4-Hydroxybenzaldehyde ester of ATP

If the chemically reactive group in the nucleotide derivative is benzaldehyde, the reactive group may form a Schiff-base with the ϵ-amino group of lysine residues in the polypeptide chain (equation 1).

$$\text{Polypeptide}-NH_2 + O{=}CH-C_6H_4-O-\text{pppA} \rightarrow \text{polypeptide}-N{=}CH-C_6H_4-O-\text{pppA} \quad (1)$$

$$\text{Polypeptide}-N{=}CH-C_6H_4-O-\text{pppA} \xrightarrow{NaBH_4} \text{polypeptide}-NH-CH_2-C_6H_4-O-\text{pppA} \quad (2)$$

$$\text{Polypeptide}-NH-CH_2-C_6H_4-O-\text{pppA} + \text{ppp*U} \xrightarrow{DNA} \text{polypeptide}-NH-CH_2-C_6H_4-O-\text{pppAp*U} + \text{pp} \quad (3)$$

These derivatives will react preferentially with amino-acid residues in the vicinity of nucleotide-binding sites on the enzyme such as the binding site within the active center of RNA polymerase. However, a strict selectivity of binding is not essential for the success of this method. In a second reaction the unstable Schiff-base is converted into a stable secondary amine by reduction under mild conditions with sodium borohydride (equation 2). Simultaneously, any excess of the aldehyde derivative is reduced to the non-reactive alcohol. So far the modified enzyme is not labeled radioactively. To avoid unspecific labeling[16] radioactivity is introduced in the next step (equation 3) where the modified enzyme is incubated with template and a single radioactive nucleoside triphosphate. Of relevance are enzyme molecules which contain the derivative covalently bound such that its nucleotide moiety is bound to the active site in the same configuration as the priming nucleotide during abortive initiation. If such a modified enzyme is still catalytically active in a template-dependent mode, then a phosphodiester bond will be formed by autocatalysis between the covalently attached nucleotide and the added radioactive second substrate. The result is a covalently bound dinucleotide which by its radioactivity firmly labels the polypeptide to which it is attached. The distance of the active center to the site of attachment cannot be larger than the length of the spacer between the nucleotide and the C-α-atom of the amino acid to which the nucleotide is bound (about 1.5–2.5 nm). Without covalent modification or reduction by borohydride, or in the absence of template no radioactive labeling should be observed. Furthermore, the oligonucleotide label should disappear after digestion with proteases but not upon incubation with deoxyribonuclease or ribonuclease.

The location of the labeled dinucleotide can be approximately determined by an elegant Maxam-Gilbert type of amino-acid specific limited proteolysis[17–19] provided the enzyme contains only one molecule of covalently bound labeled dinucleotide per polypeptide chain.

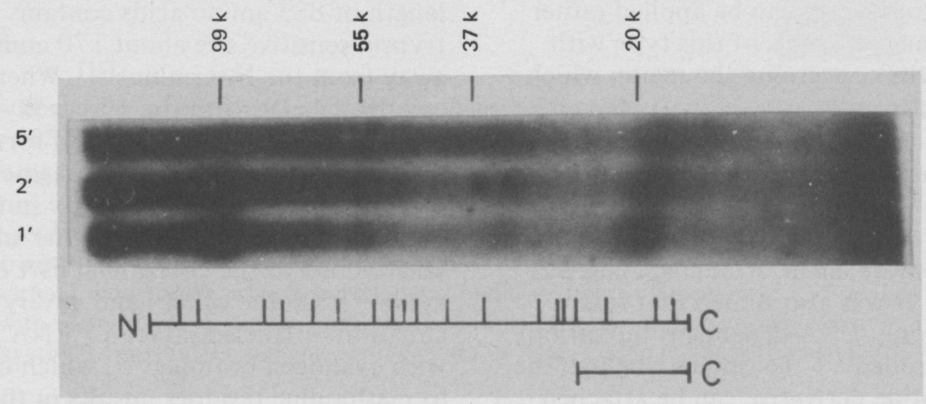


Fig. 5. Kinetics of the limited cleavage by CNBr of the affinity-labeled T7 RNA polymerase.

0.2 μg T7 RNA polymerase labeled similarly as described[21] was treated with 50mM CNBr in 50mM HCl in the presence of 0.05% dodecyl sulfate at 37 °C. Cleavage was terminated by neutralisation. The products were separated by electrophoresis in the presence of 0.1% dodecyl sulfate in a gel containing a gradient from 7.5–20% polyacrylamide. Lanes a, b, c: 1, 2, or 5 min incubation with CNBr. The migration of the fragments is from left to right (the position of marker proteins is indicated). The mobility of the most rapidly migrating spot corresponds to M_r ~20 000. The distribution of the Met-residues in the polypeptide chain is shown schematically below the autoradiography.

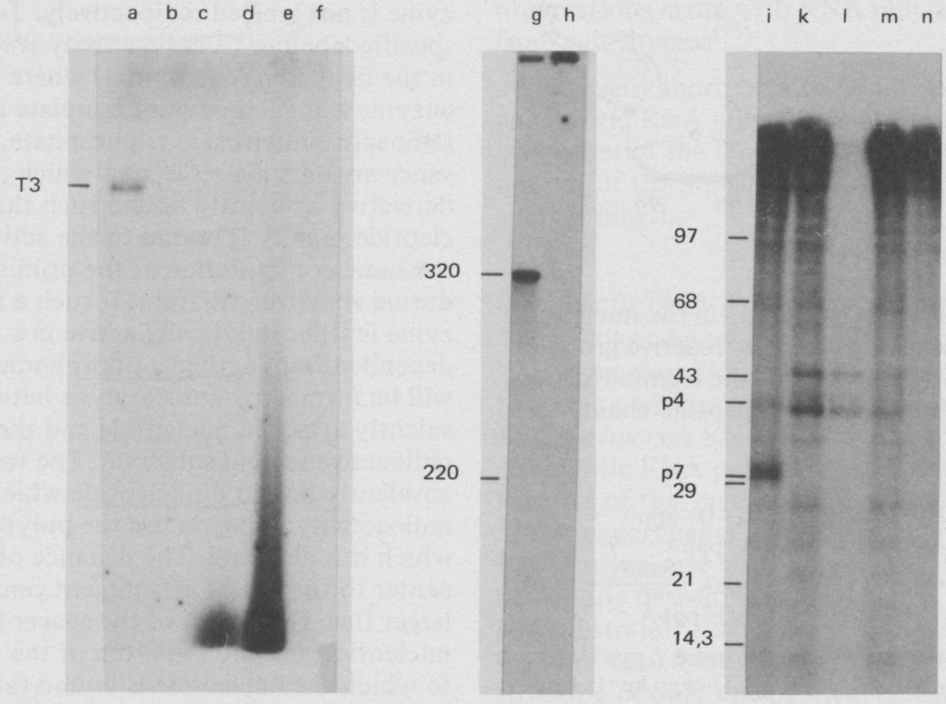


Fig. 6. Affinity labeling by autocatalysis of the RNA polymerases from bacteriophage T3 and N4.

Experiments with T3 RNA polymerase /lanes a–f): a: the incubation was carried out similarly as described[21] with 0.5 μg T3 RNA polymerase, T3 DNA and the 4-hydroxybenzaldehyde ester of GTP; b: as in a, but without derivative; c: as in a, but without DNA; d: as in a, but without reduction; e: as in b, but with added GTP; f: as in a, but with subsequent incubation with proteinase K.

Experiments with N4 virion RNA polymerase[23,24] *(lanes g and h):* g:0.66 μg enzyme was modified with 4-hydroxybenzaldehyde ester of GTP similarly as in ref.[21] but in presence of 2 μg heat-denatured N4 DNA. After reduction DNA was added again. Incubation was carried out with [α-^{32}P]ATP; h: as in g, but without derivative.

Experiments with N4 RNA polymerase II[25,26] *(lanes i–n):* 5 μg of a p4p7 preparation was modified with 4-hydroxybenzaldehyde ester of ATP similarly as in ref.[21] but in presence of heat-denatured N4 DNA. The substrate for the enzymatic reaction was [α-^{32}P]UTP; k: as in i, but without derivative; l: as in i, but without DNA; m: as in i, but without reduction; n: as in i, but followed by incubation with proteinase K.

Experiments performed during recent years have shown that this affinity labeling of RNA polymerases by autocatalysis can be applied rather generally to many enzymes of this type with interesting results concerning the region which contains the active site.

DNA-Directed RNA polymerases from phages

With T7 RNA polymerase, which contains only a single polypeptide chain, strong labeling was observed[20,21]. It was also shown that this labeling is not only DNA-dependent but strictly promoter-controlled[21]. To obtain labeling, the reactive nucleotide derivative can be attached to the RNA polymerase prior to the addition of the template. It follows that the active site is accessible for the derivative in the absence of template and, furthermore, that the correct binding of the template to the enzyme is not prevented by the covalent attachment of the nucleotide derivative.

What do we know about the position of the labeled site? The T7 RNA polymerase, with a length of 883 amino acids contains a very trypsin-sensitive site about 170 amino acids away from the N-terminus[21]. When the native enzyme labeled with the 4-hydroxybenzaldehyde ester of GTP is digested for a very short time with trypsin, this small fragment is cleaved off without loss of radioactivity in the remaining large fragment. Therefore the label cannot be attached to the N-terminal part of the enzyme[21]. On the other hand, a very short incubation of labeled intact T7 RNA polymerase with cyanogen bromide[18], which cleaves next to methionine residues, results in the formation of a small labeled polypeptide ($M_r \sim 20$ k) (Fig. 5). The enzyme contains 26 methionine residues scattered more or less evenly over the whole polypeptide chain. Therefore, the appearance of the small radioactively labeled polypeptide within a very short time of incubation indicates that the site labeled when the 4-hy-

droxybenzaldehyde ester of GTP is used as the modifying nucleotide derivative is located close to one of the two termini. Since the N-terminus has been previously excluded[21] the short labeled BrCN-cleaved fragment must be derived from the C-terminus (Fig. 5). This conclusion is supported by additional kinetic experiments based on the rapid cleavage of the two Asn-Gly bonds in T7 RNA polymerase by hydroxylamine (data not shown).

The RNA polymerase coded for by the genome of the bacteriophage T3 is closely related to the T7 RNA polymerase[22]. Therefore it is not surprising that affinity labeling of this enzyme by autocatalysis with T3 DNA as template is also achieved (Fig. 6a).

The RNA polymerase encapsulated in the virion of the bacteriophage N4 is very different from the T3 or T7 RNA polymerases[23]. The N4 virion RNA polymerase consists of a very long polypeptide chain ($M_r \sim 320$ k) and recognizes promoters with GpA or GpG RNA starting sites[24]. The genome of bacteriophage N4 codes for a second RNA polymerase, which transcribes N4 middle RNAs during the N4 life cycle. The core form of this enzyme consists of two subunits with M_r of about 30 k (p7) and 40 k (p4)[25]. Here, the transcribed RNAs start with ATP[25,26]. In each case a specific labeling of the enzyme by autocatalysis is observed (Fig. 6g and Fig. 6i–n). Of the two subunits, which constistute N4 RNA polymerase II, the poly

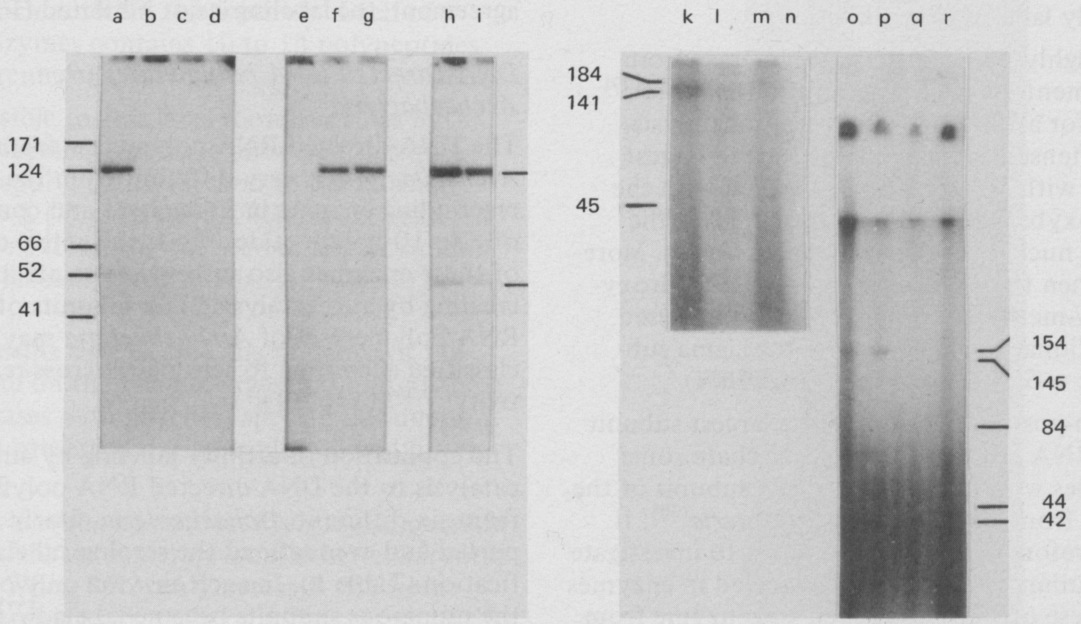


Fig. 7. Affinity labeling by autocatalysis of the RNA polymerase from the Gram-negative *Thermotoga maritima* sp. nov., *Anabaena* 7120 and the Gram-positive *Lactobacillus curvatus*.

Lanes a–i: Experiments with the enzyme from *Anabaena*[29]. a: 2 μg enzyme was modified with 0.3mM 4-hydroxybenzaldehyde ester of ATP in the presence of 1 μg plasmid pTE55 (Elliot, T., Kassavetis, G.A. & Geiduschek, E.P. (1984), cited in ref.[29]) containing a strong promoter for this RNA polymerase. After reduction the enzymatic labeling was carried out with 0.16μM [α-^{32}P]UTP; b: as in a, but without derivative; c: as in a, but without DNA; d: as in a, but without reduction; e: as in a, but followed by incubation with proteinase K; f: as in a, but followed by incubation with ribonuclease; g: as in a, but followed by incubation with deoxyribonuclease; h: as in a, but with 4-[*N*-(2′-hydroxyethyl)-*N*-methyl]aminobenzaldehyde ester of ADP; i: as in h, but with the ester of AMP as the derivative.

Lanes k–n: Experiments with the enzyme from *Thermotoga*[28] were carried out with 4-hydroxybenzaldehyde ester of ATP as the derivative as described[37], but with 40mM $MgCl_2$; the enzymatic labeling was performed at 55 °C. k: complete mixture; l: as in k, but without DNA; m: as in k, but without reduction; n: as in k, but in the presence of 100 μg/ml heparin.

Lanes o–r: Experiments with the enzyme from *L. curvatus*[32]. The experiments were carried out as described[37] with the 4-hydroxybenzaldehyde ester of ATP but with 2.5mM $MnCl_2$ and 100mM KCl instead of 10mM $MgCl_2$. Labeling was carried out at 37 °C. o: complete mixture; p: as in o, but with 2 μg/ml rifampicin; q: as in o, but without DNA; r: as in o, but without reduction.

peptide with $M_r \sim 30$ k becomes labeled. In each case all appropriate controls confirm the specific labeling.

DNA-Directed RNA polymerases from Eubacteria

Eubacteria contain a single DNA-directed RNA polymerase (M_r approximately 500 k) usually consisting of 4 to 5 polypeptide chains[27]. The first enzyme investigated with the method of affinity labeling by autocatalysis was from *Escherichia coli*[11,12]. Depending on the nature of the reactive nucleotide used, either the second largest subunit β ($M_r \sim 150$ k) or both β and σ subunits become labeled[15].

We have investigated the highly purified RNA polymerase of the extremely thermophilic Gram-negative *Eubacterium Thermotoga maritima*[28] which consists of only the core subunits β' (184 k), β (141 k) and α (45 k). As in *E. coli* the second largest subunit becomes distinctly labeled (Fig. 7k–n).

When highly purified RNA polymerase from the filamentous cyanobacterium *Anabaena*[29] is used for affinity labeling by autocatalysis, again intensive labeling of the second largest subunit with $M_r \sim 124$ k is observed with the 4-hydroxybenzaldehyde ester of ATP as the reactive nucleotide derivative (Fig. 7a–g). Moreover, when the ADP ester of 4-[*N*-(2′-hydroxyethyl)-*N*-methyl]amino benzaldehyde is used, an additional weak labeling of the sigma subunit ($M_r \sim 52$ k) is observed (Fig. 7h, i).

In Gram-positive *Eubacteria* the largest subunit of the RNA polymerase seems to share some properties with the second largest subunit of the enzyme from gram-negative *Eubacteria*[30]. It was therefore of particular interest to investigate which subunit would become labeled in enzymes from these organisms. In fact, the enzyme from Gram-positive *Eubacteria* such as *Micrococcus luteus*[31] can also be labeled by this method. After electrophoretic separation the label appears in the region of the two largest subunits (data not shown). Since the large subunits are of very similar size they are difficult to separate by gel electrophoresis in presence of dodecyl sulfate.

Separation is easier in the case of the RNA polymerase from Gram-positive *Lactobacillus curvatus* where the largest subunit ($M_r \sim 151$ k) is 4% larger than the second largest subunit ($M_r \sim 145$ k)[32]. After incubation under condition of affinity labeling, autoradiography clearly revealed that the largest subunit becomes exclusively labeled (Fig. 7o–r). This result supports the hypothesis[30] that in the RNA polymerase from Gram-positive *Eubacteria* the largest subunit fulfills the function of the second largest subunit of the enzyme from Gram-negative *Eubacteria*. Rifampicin does not block dinucleotide synthesis in *Eubacteria*[33]. In agreement, the labeling is not inhibited (Fig. 7p).

DNA-Directed RNA polymerases from Archaebacteria

The DNA-directed RNA polymerase from *Archaebacteria* is very different from the corresponding enzyme in *Eubacteria* and consist of 8 to 10 polypeptides[34]. Is the active center of these enzymes also susceptible to affinity labeling by autocatalysis? The subunits of the RNA polymerases of *Archaebacteria* may be classified according to serological cross-reactivity[35,36] (Table 1).

The application of affinity labeling by autocatalysis to the DNA-directed RNA polymerase from six different *Archaebacteria* clearly supported and even refined the serological classification (Table 1). In each enzyme only one of the numerous subunits became labeled[37]. In the enzyme from methanogenic or halophilic

Table 1. Affinity labeling of RNA polymerase from *Archaebacteria* by autocatalysis[37].

Archaebacterium	Subunit composition[a]	Labeled subunit	M_r ($\times 10^{-3}$)
Mc.[b] *vaniellii*	A B′ B″ C	B′	79
Mc.[b] *thermolithotrophicus*	A B′ B″ C	B′	79
Mb.[c] *thermoautotrophicum*	A B′ B″ C	B′	78
Halobacterium halobium	A B′ B″ C	B′	86
Archaeoglobus fulgidus	(A + C) B′ B″	B′	80
Sulfolobus acidocaldarius	B A C	B	127
Sulfolobus B12	B A C	B	127

[a] Ordered according to molecular size. Only the largest subunits are shown. The same capital letter is used for subunits which show serological cross-reactivity.
[b] *Mc.: Methanococcus.*
[c] *Mb.: Methanobacterium.*

Archaebacteria exclusively the second largest subunit B′ was labeled (Table 1). In the enzyme from the sulfate-reducing *Archaeoglobus fulgidus*[38] again only the second largest subunit B′ was labeled. In contrast, when the enzyme from two different strains of the non-methanogenic sulfur-dependent *Sulfolobus* (type BAC) was tested the largest subunit B was labeled. This clearly shows that affinity labeling by autocatalysis and immunological cross reactivity are much better evidence for functional equivalence than the molecular size of the subunits. It will be of interest to compare the sequence around the binding site of the affinity label in these various enzymes.

Eukaryotic DNA-directed RNA polymerases

Eukaryotic cells contain three different RNA polymerases designated as A (I), B (II) and C (III) which catalyse the synthesis of the large ribosomal RNAs, messenger RNAs and small RNAs such as tRNA, respectively. Each of these enzymes contains 10 to 14 polypeptides of different size.

Is it possible to also label these enzymes by affinity labeling? We have answered this question by applying this method to the purified enzymes A (I), B (II) and C (III) from yeast. In each case exclusively the second largest subunit became labeled, i.e., subunit A135, B150 or C128[13].

These results suggest that the presence of the active site on the second largest subunit of these polymerases with different specialised functions has been preserved during evolution and differentiation.

Affinity labeling by autocatalysis is also possible with the RNA polymerase from other eukaryotic sources. When RNA polymerase B (II) from wheat germ is used, again only the second largest subunit W140 becomes labeled. This is prevented by low concentrations of α-amanitin just as is the RNA synthesis catalysed by this enzyme[39]. Indeed, affinity labeling by autocatalysis requires the same catalytic activity as RNA synthesis.

To analyse the product of this affinity labeling in more detail the subunit W140 labeled with the 4-[*N*-(2′-hydroxyethyl)-*N*-methyl]aminobenzaldehyde ester of ADP and [α-^{32}P]UTP in the presence of plasmid DNA was isolated after incubation with ribonuclease and was then digested with proteinase K. The radioactive product was cleaved off with acid pyrophosphatase. Terminal phosphate groups were removed by treatment with phosphatase as described[13]. Chromatographic analysis demonstrated unequivocally that the dinucleotide ApU and the trinucleotide ApApU had been formed during affinity labeling. When the 4-hydroxybenzaldehyde ester of ATP was used, and without ribonuclease A treatment, the trinucleotides ApUpU and ApUpA were found.

For the formation of ApApU and ApUpA one would expect the presence of ATP in the reaction mixture; however, ATP had not been

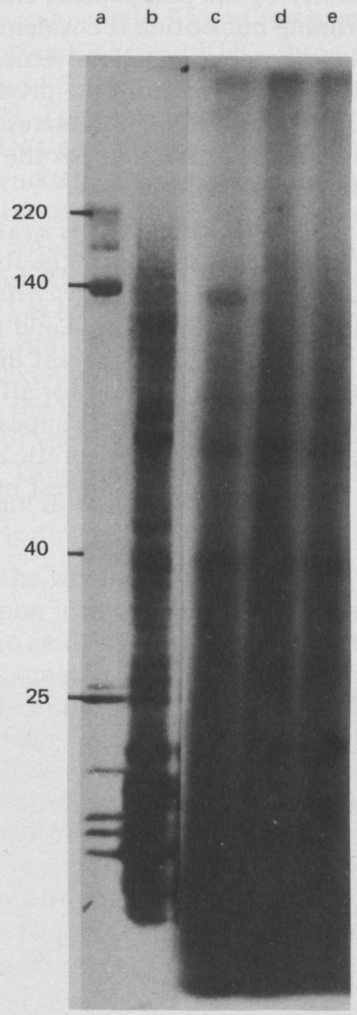


Fig. 8. Affinity labeling by autocatalysis with a crude nuclear extract from tobacco cells.

Nuclei from cultured *Nicotiana tabacum* cells were isolated[42] and broken in the Kontes mini-bomb cell disruption chamber. After addition of 0.25mM phenylmethylsulfonyl fluoride the extract was centrifuged for 30 min at 110000 x *g*. For modification the supernatant (17 μg protein) was incubated as described[13] but only for 4 min with 4-hydroxybenzaldehyde ester of ATP and, subsequently, with $NaBH_4$. The enzymic reaction was started with 3.8 μg denatured calf thymus DNA and [α-^{32}P]UTP for 4 min. Lane a: subunits of wheat germ RNA polymerase as markers; b: silver stained proteins of the supernatant; c: complete incubation mixture; d: as in c, but without derivative; e: as in c, but without reduction (c–e: autoradiography).

added. Maybe ATP existed as an impurity in the substrate or was formed by hydrolysis of the ATP derivative. Possibly, the latter could also function to a limited extent as elongating substrate[40].

Formation of trinucleotides during affinity labeling by autocatalysis has also been observed with RNA polymerase B (II) and C (III) from yeast[13].

To explain these results one has to assume a certain flexibility of the polypeptide chain to which the priming nucleotide is covalently bound to allow thereby a small movement of the dinucleotide formed within the product-binding site. Otherwise RNA polymerase must contain two adjacent active sites for the formation of two phosphodiester bonds[41].

Affinity labeling by autocatalysis is a rather sensitive method as was shown by the following experiment. Isolated nuclei from *Nicotiana tabacum* cells[42] were plasmolysed and the resulting crude 100 000 × *g* supernatant directly used without further purification for affinity labeling. Several radioactive bands appear in the autoradiography of the electrophoretic analysis (Fig. 8) but the band with the slowest mobility is absent when the nucleotide derivative or when the reduction step are omitted (Fig. 8, d, e). This band migrates at the position of the second largest subunit of RNA polymerase B (II) from *Nicotiana tabacum* RNA polymerase ($M_r \sim 135$ k)[42].

DNA Primase and Qβ Replicase

The wide applicability of affinity labeling by autocatalysis suggests that even more distantly related polymerases such as DNA primase may be susceptible to this procedure. This enzyme is a DNA-directed RNA polymerase with a rather specialised function. It synthesizes oligoribonucleotide primers required by DNA polymerase for initiation of DNA synthesis. The enzyme has been highly purified from yeast cells[44] as well as from calf thymus[45] and is strongly associated with DNA polymerase. The complex from yeast contains 5 polypeptide chains. The polypeptides with $M_r \sim 58$ k and ~ 48 k are associated with primase activity. When the method of affinity labeling by autocatalysis is applied to the complex from yeast, both subunits of primase become labeled (Foiani, M., Lindner, A.J., Hartmann, G.R., Lucchini, G. and Plevani, P., submitted for publication). The same result

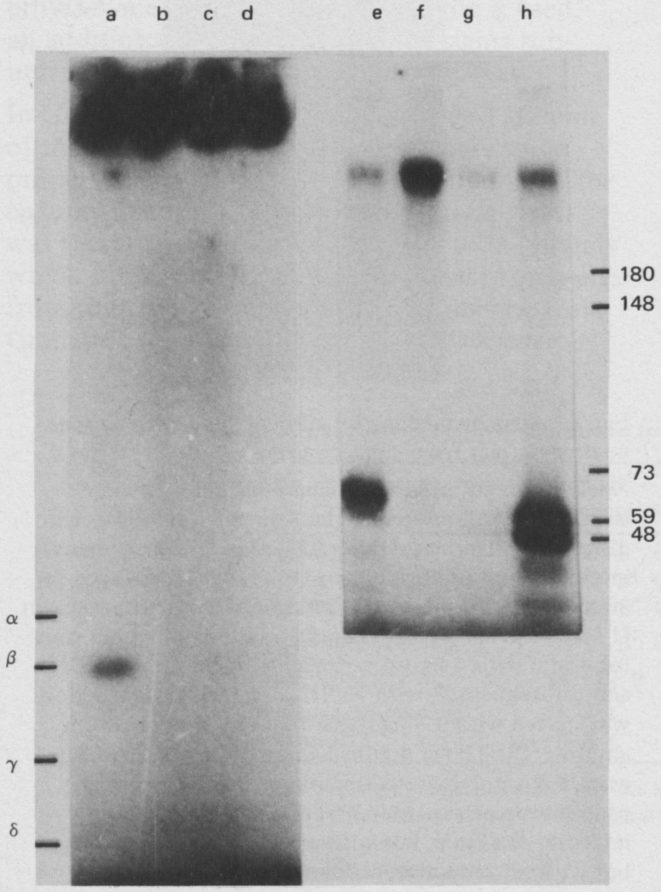


Fig. 9. Affinity labeling by autocatalysis of Qβ replicase and the complex of DNA primase and DNA polymerase from calf thymus.

Lanes a–d: Experiments with Qβ replicase (holoenzyme with the subunits α, β, γ, δ), carried out as described[37] with 1 μg enzyme[47] and 4-hydroxybenzaldehyde ester of GTP. 0.02 μg/μl MDV-I (+) RNA[47] used as template, and [α-^{32}P]GTP used as substrate. a: complete mixture; b: as in a, but without template; c: as in a, but with 100 μg/ml heparin; d: as in a, but without reduction.

Lanes e–h: Experiments with the complex of DNA primase and DNA polymerase, carried out similar as described[37] with 2 μg enzyme[45] in 10mM phosphate buffer pH 7.8 and 100 μg/ml poly(dC,dT) as template and [α-^{32}P]GTP as substrate. e: with 4-[*N*-(2′-hydroxyethyl)-*N*-methyl)aminobenzaldehyde ester of ATP; f: with the ester of ADP; g: with the ester of AMP; h: with 4-hydroxybenzaldehyde ester of ATP. The radioactive band in the upper part of the gel was not identified. This band is particularly strong with the ester of ADP, is not sensitive to digestion with proteinase K and is therefore not bound to a protein.

is found for the complex from calf thymus (Fig. 9e–h). However, the extent of labeling of the two subunits differs depending on the particular conditions applied. Frequently, the larger subunit is more heavily labeled. These observations are reminiscent of the situation found in several eubacterial RNA polymerases (Fig. 7 and ref.[15])

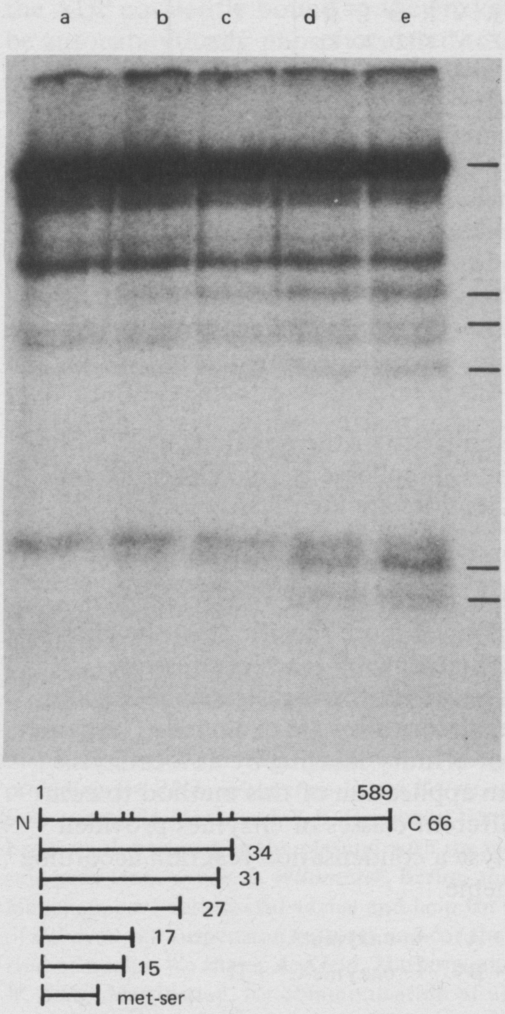


Fig. 10. Kinetics of limited cleavage by CNBr of the affinity labeled Qβ replicase β subunit.

0.2 μg electrophoretically purified labeled β subunit (Fig. 9) was dissolved in 50 μl HCl containing 1% dodecyl sulfate and then treated with 66mM CNBr at pH 1–2. Cleavage was stopped by neutralisation in presence of 2% mercaptoethanol. The products of the limited cleavage were separated by electrophoresis in a 15% polyacrylamide gel containing 0.1% dodecyl sulfate. Lane a (control): incubation for 15 min at 20 °C without CNBr; labeled bands are the products of cleavage at particularly acid labile peptide bonds[49]. b–e: incubation for 2, 5, 10 and 15 min with CNBr. The pattern of the labeled CNBr products and their size (shown) corresponds exactly to the N-terminal peptides formed by "single hit" CNBr cleavage at the met residues 323, 300, 230, 148 and 130 (Met^{76}-Ser^{77} is not cleavable under these conditions[49]) as shown in the scheme[48].

where two subunits (β and σ) become labeled. These results suggest that the two subunits of primase are associated in such a way that the covalently bound derivative can reach into the active site from attachment sites on both subunits.

Another rather specialised polymerase is the RNA-directed RNA replicase from *E. coli* bacteriophage Qβ. This RNA replicase consists of four different subunits. Three of them (α, γ and δ) are host-coded and are identical with the ribosomal protein S1 and the protein elongation factors EFT_u and EFT_s, respectively. Only subunit β, is coded for by the viral genome[46]. The enzyme selectively transcribes specific RNA templates with a cytidine cluster at the 3′-end which acts as the starting site for RNA synthesis. Evidently Qβ replicase is not a general RNA-directed RNA polymerase. With midivariant RNA (MDV-1, 221 nucleotide length[47]) as template and [α-^{32}P]GTP as substrate the viral coded subunit β ($M_r \sim 66$ k[48]) becomes exclusively labeled. As in all previous examples the labeling is dependent on the presence of the derivative as well as on the template. Labeling is prevented by 100 μg/ml heparin and does not occur when the reduction step is omitted (Fig. 9a–d).

Where is the label attached to the polypeptide chain? Upon inspection of the sequence of the 589 amino acids of the β subunit from the replicase it becomes evident that methionine residues occur only in the N-terminal half of the polypeptide chain[48]. Cleavage of the labeled polypeptide chain with cyanogen bromide for a short time under conditions where only one cleavage per polypeptide chain should occur[17–19,49] leads to the appearance of two relatively small fragments with M_r of about 17 k and 14 k, respectively (Fig. 10). The labeled double band corresponds in length to the expected cleavage products at positions 148 and 130 of the 589 amino acid long sequence. This result suggests that the labeled lysine residue occurs within the first 130 amino acids near the N-terminus of the polypeptide chain.

Sequence homologies at the binding site

The surprising fact that such a large variety of different RNA polymerases can be labeled by the same method suggests that all labeled polypeptides should contain a similar amino-acid sequence at or close to the active site. The benzaldehyde derivatives used in our experiments are most likely attached to the ε-amino group of a lysine residue as has been suggested for the RNA polymerase from *E. coli*[15,18].

Table 2. Comparison of the amino-acid sequence between Lys^{1048} and Arg^{1058} in the β subunit of *E. coli* RNA polymerase with a similar region in labeled polypeptides from other RNA polymerases.

The subunit, its approximate molecular mass, the position of the first amino acid of the given sequence and the source of the sequence is shown.

			Lit.
E. coli β (150 k)	1048	K I V K V Y L A V K R	(50)
Yeast B140	962	K F V K V R V R T T K	(52)
Mb. thermo.[a] B' (78 k)	345	R L A K I R V R E Q R	[d]
S. acido.[b] B (126 k)	859	K L V K V R V R D L R	[e]
T3 (T7) (98 k)	704	K L L A A E V K D K K	(22)
Qβ β (66 k)	95	K F L A A E A –[c] E C A	(48)
Yeast primase (48 k)	135	K F I S L A M K I T N	(53)

[a] *Methanobacterium thermoautotrophicum* strain Winter.
[b] *Sulfolobus acidocaldarius*.
[c] Assumed deletion.
[d] Berghöfer, B., Schallenberg, J. & Klein, A., Fachbereich Biologie-Molekulargenetik, Universität, D-3550 Marburg, personal communication.
[e] Pühler, G. & Zillig, W., Max-Planck-Institut für Biochemie, D-8033 Martinsried, personal communication.

Depending on the nature of the nucleotide derivative used, the labeled oligonucleotide is covalently attached in this enzyme to either one of two adjacent regions of the β-subunit[50]. One is located between Ile^{1036} and Met^{1066}[18], the other between Met^{1232} and Met^{1243}[19]. When searching for similar sequences among the labeled RNA polymerase polypeptides with known primary structure such regions are indeed found (Tables 2 and 3). They differ mostly in conservative substitutions.

Particularly striking is the similarity of these regions between eukaryotic and archaebacterial RNA polymerases. It is also interesting to note that even so different polymerases as Qβ replicase and T7 or T3 RNA polymerase contain similar sequences. Compared at the nucleotide level the similarity of these regions in T7 polymerase and Qβ replicase is even closer. 16 out of 19 nucleotides are identical.

Outlook

Affinity labeling of the active site by autocatalysis is much more specific than the classical methods of labeling by reactive substrate analogues because other substrate binding sites such as regulatory sites are excluded. The general nature of affinity labeling by autocatalysis suggests an application of this method to completely different classes of enzymes provided they catalyse a condensation reaction according to the scheme

enzyme + A → enzyme-A
enzyme-A + B* → enzyme-C* + D

Table 3. Comparison of the amino-acid sequence between Leu^{1233} and Lys^{1242} in the β subunit of *E. coli* RNA polymerase with a similar region in labeled polypeptides from other RNA polymerases.

The subunit, its approximate molecular mass, the position of the first amino acid of the given sequence and the source of the sequence is indicated.

			Lit.
E. coli β (150 k)	1233	L K L N H L V D D K	(50)
Yeast B140		Q R L R H M V D D K	(52)
Mb. thermo.[a] B' (78 k)	481	Q K L H H M T T D R	[c]
S. acido.[b] B (126 k)	958	L K L G H L P D S T	[d]

[a] *Methanobacterium thermoautotrophicum* Winter.
[b] *Sulfolobus acidocaldarius*.
[c] Berghöfer, B., Schallenberg, J. & Klein, A., Fachbereich Biologie – Molekulargenetik, Universität, D-3550 Marburg, personal communication.
[d] Pühler, G. & Zillig, W., Max-Planck-Institut für Biochemie, D-8033 Martinsried, personal communication.

in two separate steps. The specificity for the priming substrate A should not be too narrow to allow chemically reactive derivatives to be used. The second substrate B should be radioactively labeled in such a way that the radioactivity remains in the product of condensation. An example of this type is creatine kinase which can be modified covalently by periodate-oxidised ADP at its active center. It was shown that the ADP covalently bound to the enzyme can be autocatalytically phosphorylated to ATP by creatine phosphate[51]. There is no doubt that many other examples can be found where it is possible to localise the region containing the active-center through affinity labeling by autocatalysis. In view of the fact that an increasing number of enzyme primary structures will be known by gene sequencing without concomitant knowledge of the active site, affinity labeling by autocatalysis with subsequent amino-acid specific limited proteolysis will become an increasingly useful technique to determine the region containing the active site. A particular advantage is the requirement of only tiny amounts of not necessarily pure enzyme.

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