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*ASYMMETRIC RNA SYNTHESIS IN VITRO: HETEROLOGOUS DNA-ENZYME SYSTEMS; E. COLI RNA POLYMERASE**

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The synthesis of RNA in living cells is so restricted and regulated as to produce patterns of gene expression characteristic of the organism, its time, and its environment. An aspect of this regulation which has received some attention during the past three years is that of strand selection.[1-14]

Recently, some of us described the ability of *B. megatherium* extracts to use selectively one strand of the DNA of its bacteriophage α as a template for RNA synthesis, excluding the other strand.[14] The RNA produced in this *in vitro* system is asymmetric. Its interactions with αDNA and the purified purine-rich ("light") strand of αDNA are the same as those of pulse-labeled RNA from phage α-infected *B. megatherium*. We had shown that the complete integrity of phage DNA is not essential for such asymmetric synthesis, but that its ordered helical conformation is. In this communication we present further properties of this and related

systems in which the asymmetric synthesis of RNA on a variety of viral DNA templates can be demonstrated.

Materials and Methods.—*B. megatherium* (Paris strain), *B. subtilis* 168, *B. cereus*, *A. aerogenes*, and *Ps. fluorescens* were grown as overnight cultures in nutrient broth, centrifuged, washed, and stored at −20°C. *E. coli* were bought as a frozen paste from Grain Processing Corp., Muscatine, Iowa. Lyophilized *M. lysodeikticus* cells were purchased from the Miles Chemical Company.

Two types of bacterial extracts were prepared: (1) supernatant fractions were prepared as described previously by grinding bacteria with alumina and centrifuging 2–2½ hr at 90,000 *g* in the cold; (2) $(NH_4)_2SO_4$ fractions were prepared from these supernatants at 0°C by adding the solid salt. The material precipitating at 40 or 60% saturation was collected and redissolved in 0.01 *M* Tris, pH 7.9, 0.01 *M* $MgCl_2$, 10^{-4} *M* EDTA.[25]

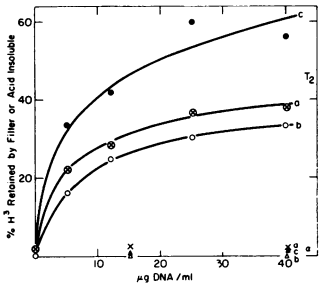

FIG. 1.—Comparison of three different assays of DNA-RNA homology. T2 RNA synthesized asymmetrically with *E. coli* RNA polymerase is annealed with T2 and αDNA for 6 hr at 60°C in 2 SSC. RNA concentration in the annealing mixture, 0.65 μg/ml. Aliquots of each annealed sample are taken for (*a*) digestion for 15 min at 37°C with 5 μg pancreatic RNase/ml and 0.5 μg T1 RNase/ml in 0.5 ml SSC + 0.1 *M* Tris, pH 7.5 followed by TCA precipitation; (*b*) RNase digestion (as above) followed by filtration through nitrocellulose membranes; (*c*) filtration through nitrocellulose without prior RNA degradation. Results of annealing with T2 DNA: ⊗, ○, ●. Results of annealing with αDNA: ×, △, ▲.

Preparation of RNA labeled with CTP-H³ and assays of DNA-RNA homology were performed as described previously.[14] *E. coli* RNA polymerase was prepared after the method of Chamberlin and Berg.[2] *M. lysodeikticus* RNA polymerase was prepared after the method of Nakamoto, Fox, and Weiss,[15] and purified to the $(NH_4)_2SO_4$ or DEAE[25] step. Both types of enzyme preparations had the same transcription properties. We are grateful to S. B. Weiss for gifts of some of this material. DNA was extracted from bacteriophages SP01, α, T7, N1, B3, and T2 with phenol. Two methods of testing asymmetric RNA were used: RNA-DNA homology with α and α_LDNA and RNA-RNA self-complementarity. The former method permits one to work with crude systems that utilize both endogenous and exogenous DNA templates for RNA synthesis. On the other hand, when symmetric or asymmetric RNA is predominantly synthesized on one particular template, then one may also use the property of RNA self-annealing,[4] and free one's self of the limitation of working with those DNA's whose complementary species can be isolated and separated.

DNA-RNA homology was measured by retention of annealed DNA-RNA hybrids on nitrocellulose filters after RNase digestion[16, 17] as described previously.[13] This permits measurements against low backgrounds. However, the following experiment suggests that it underestimates the amount of RNA homologous to a given DNA:[17] T2 RNA is annealed to various concentrations of T2 and αDNA and analyzed for retention on nitrocellulose filters with and without RNase digestion, as well as for TCA precipitability after RNase digestion. The data are shown in Figure 1. The level of artifact interaction with heterologous αDNA is seen to be low when detected either with or without RNase digestion. On the other hand, the estimates of annealing of T2 RNA with T2 DNA, with and without RNase digestion, are very different (Fig. 1, curves *b* and *c*). Clearly, the RNase is able to remove, by digestion, material which is shown to be capable of binding specifically to the homologous DNA. The near-coincidence of TCA and filtration assays after RNase treatment (curves *a* and *b*) shows that the effects of RNase on homologous RNA-DNA mixtures is not merely one of disaggregation. The RNase-susceptible material retained by the filters could be (1) RNA not made on a DNA template, covalently linked to RNA that has been made on a DNA template; (2) RNA made by imperfect replication mechanisms involving, say, slippage or repetitive transcription of limited segments of DNA template. The annealing of such material to DNA would be imperfect; (3) perfectly transcribed but imperfectly annealed RNA.[18] Category 1 is not likely to comprise a significant fraction of the synthesized RNA. On the other hand, alternative 2 might be, and 3 must be, included within the

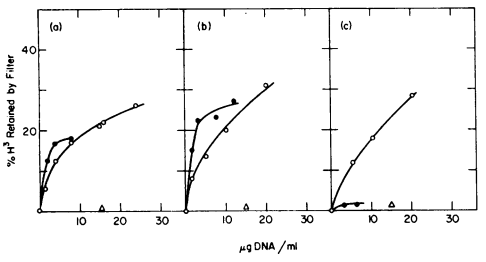

FIG. 2.—Interaction of αRNA with α, α_L, and T2 DNA.

(*a*) RNA synthesized on a template of denatured αDNA in a *B. megatherium* extract. Synthesized RNA is at a concentration of 1.0 μg/ml (Fig. 7*a* of ref. 14).

(*b*) RNA synthesis on a template of native αDNA catalyzed by purified *M. lysodeikticus* polymerase. RNA concentration 0.8 μg/ml (Fig. 3*a* of ref. 14).

(*c*) RNA synthesized on a template of native αDNA in a *B. megatherium* supernate. RNA concentration 1.6 μg/ml (Fig. 2 of ref. 14).

Conditions of annealing: 6 hr at 60°C in 2 SSC. ○, αDNA; ●, α_LDNA; △, T2 DNA.

definition of RNA homologous to a given viral DNA template. In this sense, our assay of DNA-RNA annealing by retention on nitrocellulose filters after RNase digestion is an underestimate and we shall frequently cite analyses with and without RNase digestion.

RNA self-complementarity was assayed by measuring RNase resistance after annealing varying concentrations of RNA for 6 hr at 60°C in 2 SSC. RNase digestion was done at 37°C for 15 min in SSC + 0.1 *M* Tris, pH 7.5, using 5 μg pancreatic RNase and 0.5 μg T1 RNase in 0.5–0.7 ml of assay solution. Samples were then precipitated with TCA and counted on nitrocellulose filters as described previously.[4] The qualitative agreement obtained when extent of asymmetry is determined by RNA-DNA hybrid formation, and by RNA-RNA self-annealing, is demonstrated in Figures 2 and 3. Figure 2 shows the extent of annealing of three RNA preparations with αDNA and α_LDNA: (*a*) the RNA synthesized when denatured αDNA serves as a template in megatherium extract; (*b*) the symmetric RNA that is made when αDNA serves as template for purified *M. lysodeikticus* polymerase; and (*c*) the asymmetric RNA that is made when native αDNA serves as the template for unfractionated *B. megatherium* supernatant. The pattern of self-annealing of these RNA preparations is shown in Figure 3 (curves *b* and *c*). The qualitative distinction between these asymmetric and symmetric RNA preparations can be drawn by the use of either technique.

Experiments and Discussion.—The system in which we previously found asymmetric RNA synthesis[14] is an homologous one—an extract of uninfected *B. megatherium* acting on the DNA of one of its phages. It resembles, in this respect, the other systems in which asymmetric[12] or otherwise restricted[23] RNA synthesis has been demonstrated. We were interested in seeing to what extent homology of extract and DNA was an absolute requirement. For this purpose we designed a series of experiments in which various bacterial extracts were used with a given DNA template and another series in which a given bacterial extract was used with various phage DNA templates. Using an αDNA template, it was found that ribosomal supernatant fractions from a variety of bacteria synthesize at least partially asymmetric RNA (Fig. 4). All of the extracts incorporate ribonucleoside triphosphates, to varying extents, into TCA-insoluble material in the absence of added DNA; some (Aerobacter, Pseudomonas) can be only slightly stimulated by added DNA. Nevertheless, a substantial portion (Fig. 4) of the RNA that is synthesized by each extract, anneals specifically with αDNA, and therefore serves our purpose. These extracts make much less α_LDNA-complementary than total αDNA-complementary RNA. The relative extent of annealing with α and α_LDNA varies from preparation to preparation but (cf. Figs. 2*b* and *c*) all demonstrate pronounced asymmetry using heterologous systems of enzyme and DNA. Similarly, other DNA's can serve as templates for the synthesis of asymmetric RNA. In one series of experiments $(NH_4)_2SO_4$ fractions of *Ps. fluorescens* were used, whose GTP^{32}-incorporation could be stimulated two- to threefold by added salmon DNA. The

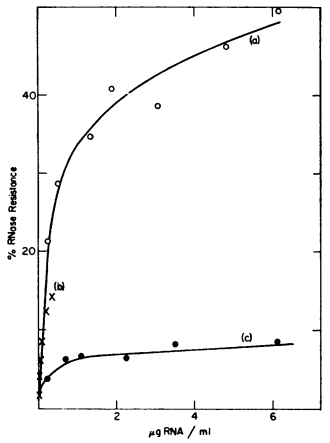

FIG. 3.—Self-complementarity of αRNA. Acquisition of RNase resistance by RNA as a result of self-annealing. The samples are described in Fig. 2. Conditions of annealing as in Fig. 2; analysis described in *Methods*. The "zero concentration" sample is one that has been heated (at some suitable concentration) to 100°C in 0.01 *M* Tris, pH 7.5 (or more dilute buffer) for 5–10 min and quenched just prior to analysis.

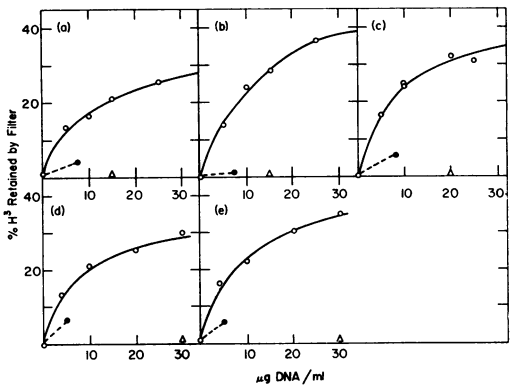

FIG. 4.—Interaction of RNA, made on αDNA templates in various bacterial extracts, with α (○), α_L (●), and T2 (△) DNA.

(*a*) *B. subtilis;* synthesized RNA is at a concentration of 0.45 μg/ml in the annealing mixture.

(*b*) *B. cereus;* RNA concentration 0.85 μg/ml.

(*c*) *E. coli;* RNA concentration 0.7 μg/ml.

(*d*) *Ae. aerogenes;* RNA concentration 0.4 μg/ml.

(*e*) *Ps. fluorescens;* RNA concentration 0.5 μg/ml.

Conditions of annealing and analysis as in Fig. 2 and *Methods*.

synthesized RNA, whose homology with the added DNA template was measured, was analyzed by RNA-RNA self-annealing. Substantially asymmetric RNA synthesis could be demonstrated for SPO1 (a *B. subtilis phage*) (Fig. 5, curve *b*), and T2 (Fig. 5, curve *c*), as well as α (Fig. 5, curve *a*) DNA. Other combinations of crude enzyme and DNA have also yielded asymmetric RNA (e.g., Fig. 5, curve *d*). The possibility of asymmetric RNA synthesis in heterologous systems is clearly established by these experiments.

We turn next to a series of experiments in which special attention was given to *E. coli* polymerase. This is the enzyme which has been the subject of more inquiries regarding asymmetric and restricted RNA synthesis than any other. We find that T2 DNA serves as a template for its asymmetric RNA synthesis (Fig. 6, curve *a*). Helical DNA is required (Fig. 6, curves *a* and *b*). The self-annealing of symmetric T2 RNA is shown for comparison (Fig. 6, curve *c*), and the asymmetry of the RNA made when T2 is used in the *B. megatherium* extract is shown in curve *d*. In all four preparations, a large fraction of the synthesized RNA anneals with T2 DNA, so that the self-annealing assays must reflect properties of the T2 RNA.[20]

It seems interesting to follow this property of the *E. coli* RNA polymerase during the course of purification. It is present in the crude ribosomal supernate (αDNA, Fig. 4, curve *c*), persists after much of the nucleic acid has been removed by $(NH_4)_2SO_4$ or streptomycin precipitation (data not shown), and remains in more highly purified $(NH_4)_2SO_4$ or DEAE fractions (Fig. 6, curve *a*). Hence, asymmetric transcription is not a property that is gained by the enzyme as it is freed of contaminant proteins such as nucleases, but is there to begin with. However, not all DNA templates yield asymmetric RNA with *E. coli* polymerase. The transcription of SPO1 and T7 DNA (Fig. 7), though still substantially asymmetric, is distinctly

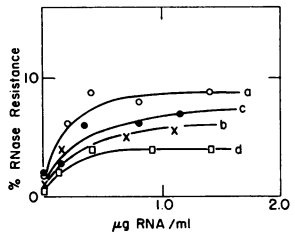

FIG. 5.—Self-complementarity of RNA synthesized on various DNA templates with a 0–60% $(NH_4)_2SO_4$ *Ps. fluorescens* fraction. Conditions of RNA synthesis as previously described[14] but using 2.5 μmoles/ml $MnCl_2$ instead of 10 μmoles $MgCl_2$/ml. Conditions of analysis as in Figs. 2 and 3 and *Methods*.

(*a*) αDNA: 0.09 μmoles (nucleotide) RNA synthesized in 15 min at 31°C per μmole template DNA. 56% of the synthesized RNA anneals with a 130-fold excess of αDNA as judged by retention on NC filters *after* RNase digestion.

(*b*) SPO1 DNA: 0.025 μmoles RNA synthesized/μmole template DNA. 74% of the synthesized RNA anneals with a 100-fold excess of SPO1 DNA as judged by retention on filters before RNase digestion (55% after RNase).

(*c*) T2 DNA: 0.07 μmoles RNA synthesized/μmole template DNA. 23% of the synthesized RNA anneals with a 130-fold excess of T2 DNA as judged by retention on filters after RNase digestion.

(*d*) Self-complementarity of SPO1 synthesized with a 0–60% $(NH_4)_2SO_4$ *B. subtilis* fraction. Conditions of RNA synthesis and analysis as above. 0.08 μmoles RNA synthesized/μmole DNA template. 65% of the synthesized RNA anneals with an 80-fold excess of SPO1 DNA, before RNase digestion (53% after RNase).

less so than that of T2 DNA (Fig. 6, curve *a*). That of N1 and B3 DNA, the hosts of which are *M. lysodeikticus* and *Ps. fluorescens*, respectively, fails to show asymmetry sufficiently pronounced to be detectable by the methods used here. We do not yet know what it is that distinguishes these DNA's prepared in similar ways and offering no obvious sign of DNA disorder.

Comments.—RNA synthesis, *in vivo*, is assumed to be initiated at defined chromosomal loci and to proceed in a unique direction relative to the transcribed DNA strand. The extent to which an *in vitro* system displays these same properties determines its suitability for a variety of purposes. What inferences about these properties of an RNA-synthesizing system can be drawn from observations on the asymmetry of RNA? Four possible situations may be considered as plausible for an *in vitro* system: (1) Synthesis may be initiated at any (or very many) location(s) on either DNA strand and proceed in a defined direction. In a macroscopic system, after any arbitrary period of synthesis, all possible complementary DNA sequences are thus transcribed, although the length of the RNA chains in which they are contained is time-dependent. The RNA is symmetric, capable of self-annealing. (2) Only one of the complementary DNA strands serves as a transcription template. Whether initiation of RNA polymerization on the transcribing strand is restricted or not, the product RNA is totally asymmetric. (3) RNA synthesis may be initiated at a limited number of sites on both the complementary DNA chains. The asymmetry of the RNA product depends on the extent of synthesis. For sufficiently limited synthesis, the product may be completely asymmetric even in the absence of termination sites. (4) RNA synthesis can only be initiated in part of the chromosome. The product, whether symmetric or asymmetric, is unable to hybridize with portions of the DNA offered as template. Asymmetry analysis by itself therefore serves mainly to identify initiation of RNA synthesis at restricted loci. Auxiliary information, derivable, for example, from quantitative study of DNA-RNA annealing[17] and kinetics of RNA synthesis,[21] is needed to determine the frequency of initiation sites, the distribution of RNA species, and their relationship to messenger RNA.

In these terms, the currently available data regarding the mode of action of various RNA-polymerizing systems is the following: *M. lysodeikticus* RNA polymerase

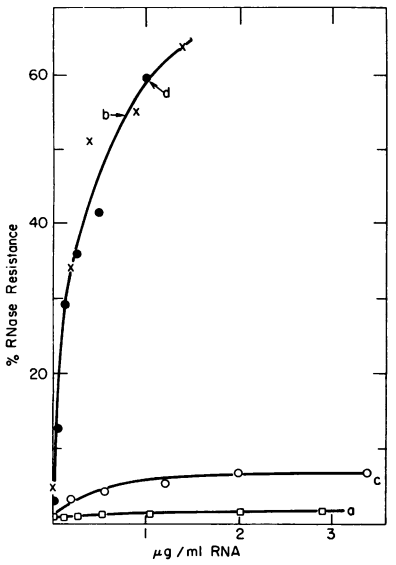

FIG. 6.—Self-complementarity of T2 RNA. Acquisition of RNase resistance by RNA as a result of self-annealing. Conditions of annealing as in Fig. 2; analysis described under *Methods*. The "zero concentration" sample is one that has been heated as described in Fig. 3.

(*a*), □: T2 RNA synthesis on native T2 DNA template catalyzed by *E. coli* RNA polymerase (DEAE fraction; #4 of ref. 2).

(*b*), ×: RNA synthesis on denatured T2 template catalyzed by *E. coli* RNA polymerase [$(NH_4)_2SO_4$ fraction; #3 of ref. 2].

(*c*), ●: RNA synthesis on native T2 DNA template catalyzed by *M. lysodeikticus* RNA polymerase.[15]

(*d*), ○: RNA synthesis on native T2 DNA template in *B. megatherium* extract. 38% of the synthesized RNA anneals with a 90-fold excess of T2 DNA after RNase digestion, as judged by retention on NC filters.

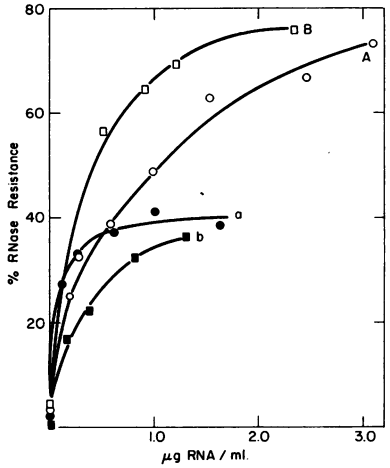

FIG. 7.—Partial asymmetry of T7 and SPO1 RNA synthesized with *E. coli* polymerase (DEAE fraction prepared by M. Hayashi according to ref. 12; it transcribes T2 DNA asymmetrically). Comparison with symmetric RNA. Annealing and analysis as described in Figs. 2 and 3, and under *Methods*.

(*a*) SPO1 RNA 0.08 μmoles RNA synthesized/μmole DNA template in 15 min at 31°C. (*A*) [comparison], symmetric SPO1 RNA synthesized with *M. lysodeikticus* RNA polymerase.

(*b*) T7 RNA 0.08 μmoles RNA synthesized/μmole DNA template in 15 min at 31°C. (*B*) [comparison], symmetric T7 RNA synthesized with *M. lysodeikticus* RNA polymerase.

purified according to the method of Nakamoto, Fox, and Weiss apparently functions according to a mechanism consistent with scheme 1, since the product appears to be essentially symmetric, whatever the detailed conditions of synthesis or the nature of the templates.[15, 19] The situation with respect to *E. coli* RNA polymerase is somewhat more confused. Initial tests of symmetry involved the use of synthetically replicated double-stranded φX174 DNA as template and showed symmetric synthesis[1, 2] (scheme 1). The significance of this result is not now clear. Hayashi and Spiegelman subsequently made the discovery that a highly purified *E. coli* polymerase could be prepared that copied only one strand of φX174 replicative form DNA (scheme 2). Other highly purified fractions were found to copy both strands.[12] These collaborators also found that the asymmetry of synthesis was stringently dependent on the DNA secondary structure. The *in vitro* replicated DNA that had been used as a template in the first experiments is, in this regard, an uncertain material that may, under certain conditions of synthesis, have a complex structure.[20, 22]

Khesin and co-workers[23] last year claimed, on the basis of competitive RNA-DNA annealing experiments, that T2 DNA served as template for restricted RNA synthesis with relatively crude *E. coli* extracts. The asymmetric T2 RNA synthesis reported here also requires that initiation of RNA synthesis on the DNA template be restricted (scheme 2 or 3). The mechanism of asymmetric synthesis of αRNA[14] in various systems is, like that of ϕX174,[12] additionally defined by the availability of fractionated DNA strands and is consistent only with scheme 2.

The experiments reported herein contribute the following information regarding a variety of purified and crude DNA-dependent RNA polymerizing systems: (1) Asymmetric transcription of αDNA occurs not only with the homologous *B. megatherium* extracts but with supernatant preparations of a number of unrelated bacterial species. (2) Other DNA templates also yield asymmetric RNA in bacterial supernatant preparations. (3) Asymmetric transcription of T2 and, to some extent, other DNA's is a property of *E. coli* RNA polymerase at various stages of purification. Clearly, this property is one that is present to begin with, not one that is acquired at the last stages of purification. In comparing these experiments with the prior studies of *E. coli* polymerase and messenger RNA synthesis in *E. coli*, one would postulate that the property can be lost. However, the conditions under which this occurs are undefined. Possibly, different DNA templates show the results of different stages in this modification of the enzyme.

One may summarize our current understanding of asymmetric RNA synthesis broadly as follows: the asymmetric *in vitro* synthesis of RNA can be shown to occur in a considerable variety of systems not limited by homology. Like its *in vivo* counterpart, it involves the interaction of enzyme with limited numbers of initiation sites on DNA templates. However, in no instance, thus far, has the identity of these initiation loci in corresponding *in vitro* and *in vivo* systems been established. The ordering of the DNA secondary structure is essential for this restricted synthesis.[14] Evidently, base interactions in the DNA double helix modulate the DNA-enzyme interactions, so as to restrict the initiation of RNA polymerization. These template-enzyme interactions are also sensitive to changes in the protein, more sensitive, in the *E. coli* system, than the polymerase activity itself.[12] Possible models for these interactions and their changes abound in current work.[24] The precise mechanism remains to be elucidated.

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[25] The following abbreviations are used: EDTA, sodium ethylenediaminetetraacetate; Tris, tris(hydroxyamino) methane; RNase, ribonuclease; CTP-H^3, cytidine 5′ triphosphate, H^3 labeled; GTP^{32}, guanosine 5′ triphosphate, P^{32} labeled in the ribose-proximal phosphate; α_LDNA, the purified, purine-rich strand of phage αDNA; SSC, "standard" saline citrate—0.15 *M* NaCl 0.015 *M* Na citrate; NC, nitrocellulose; DEAE, diethylaminoethyl-cellulose.

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*ON THE MECHANISM OF ACETYLATION OF FETAL AND CHICKEN HEMOGLOBINS**

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The discovery of N-acetylated amino acids[1, 2] at the terminals of a number of proteins naturally focused attention on the mechanism by which this acetyl group becomes attached. Pearlman and Bloch[3] stimulated interest by a study related to the transfer of acetyl amino acid to sRNA using the supernatant fraction of calf liver. Narita *et al.*,[4] using a similar preparation from rat liver, could not find such a transfer.[2] They explain the activity of some acetyl amino acids in promoting ATP-pyrophosphate exchange[2] by the presence in liver extract of a deacetylase which liberates the free amino acids. Similar experiments done by us with calf liver extracts confirm the negative results of Narita as well as the presence of an active