enzyme reactions and consequently metabolic flux. They suggested that an important aspect of enzyme evolution may be related to changes in protein structure that alter metabolite transfer within a pathway. Perhaps genetic variation in the LDH-B isozyme of the mummichog affects its association with other glycolytic enzymes and thereby affects the rate of glucose use.

Evidence from a variety of other animals and enzyme systems has also indicated that allozyme variation is associated with metabolic and organismal level differences that may affect fitness (*5–17, 20*). However, our experiments suggest that the organismal differences correlated with the *Ldh-B* polymorphism in the mummichog are causally linked. If this is not a unique observation, allozymic variation may be important in the evolution of species.

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Related RNA Polymerase–Binding Regions in Human RAP30/74 and *Escherichia coli* σ70

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RAP30/74 is a heteromeric general transcription initiation factor that binds to mammalian RNA polymerase II. The RAP30 subunit contains a region that is similar in amino acid sequence to the RNA polymerase–binding domain of the *Escherichia coli* transcription initiation factor sigma 70 (σ70). Mammalian RNA polymerase II specifically protected a serine residue in the σ70-related region of RAP30 from phosphorylation in vitro. In addition, human RAP30/74 bound to *Escherichia coli* RNA polymerase and was displaced by σ70. These results suggest that RAP30 and σ70 have functionally related RNA polymerase–binding regions.

RNA POLYMERASE II REQUIRES several general factors for initiation of transcription at the promoters of protein-encoding genes (*1*). Although many of these factors have been purified extensively, the functions of most of them in the initiation of transcription are not well characterized. RAP30/74 (RNA polymerase–associated protein), also known as TFIIF (*2*), is a heterodimeric general transcription initiation factor that binds to eukaryotic RNA polymerase II (*3, 4*). An adenosine triphosphate (ATP)–dependent DNA helicase activity that associates with RAP30/74 may be related to its function (*5*). The amino acid sequence of RAP30 deduced from its cDNA (*5*) shows that RAP30 contains a region with sequence similarity to a domain of the bacterial transcription initiation factor σ70. This domain of σ70 is conserved throughout the σ factor family and is required for the binding of σ70 to RNA polymerase (*6–8*). The structural similarity between RAP30 and σ70 suggests that RAP30 could contain an RNA polymerase–binding region that is functionally related to that of σ70. The demonstration of functional similarity between RAP30/74 and σ70 would suggest the use of the well-characterized prokaryotic transcription initiation system as a paradigm for initiation factor function in higher organisms.

In order to identify a region of RAP30/74 that is associated with RNA polymerase II, we used the catalytic subunit of cyclic adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (pKA) as a reagent to modify RAP30/74 and assessed the ability of RNA polymerase II to protect RAP30/74 from phosphorylation by pKA (*9*) (Fig. 1). RAP30/74 that had been purified by RNA polymerase II affinity chromatography (*3*) was phosphorylated in vitro with pKA. The RAP74 subunit was phosphorylated ten times as efficiently as RAP30. RAP30 was then immunoprecipitated after denaturation of the RAP30/74 heterodimer (Fig. 1A, lane 5). Excess RNA polymerase II in the reaction blocked phosphorylation of RAP30 (lane 6). RNA polymerase II did not affect phosphorylation of

Table 1. Approximate molar dissociation constants for RAP30 and σ70 binding to RNA polymerase.

Protein	*E. coli* RNA polymerase	Mammalian RNA polymerase II
σ70	$<3 \times 10^{-10}$*	$>10^{-6}$ †
RAP30	$<3 \times 10^{-8}$ ‡	2×10^{-8} §

*In (*28*). †Estimated from the partial cosedimentation of σ70 with 4×10^{-7} M RNA polymerase II (Fig. 2F). ‡Estimated from the complete cosedimentation of RAP30/74 with 4×10^{-7} M RNA polymerase (Fig. 2C) and the poor displacement of RAP30/74 from RNA polymerase by NusA. §In (*3*).

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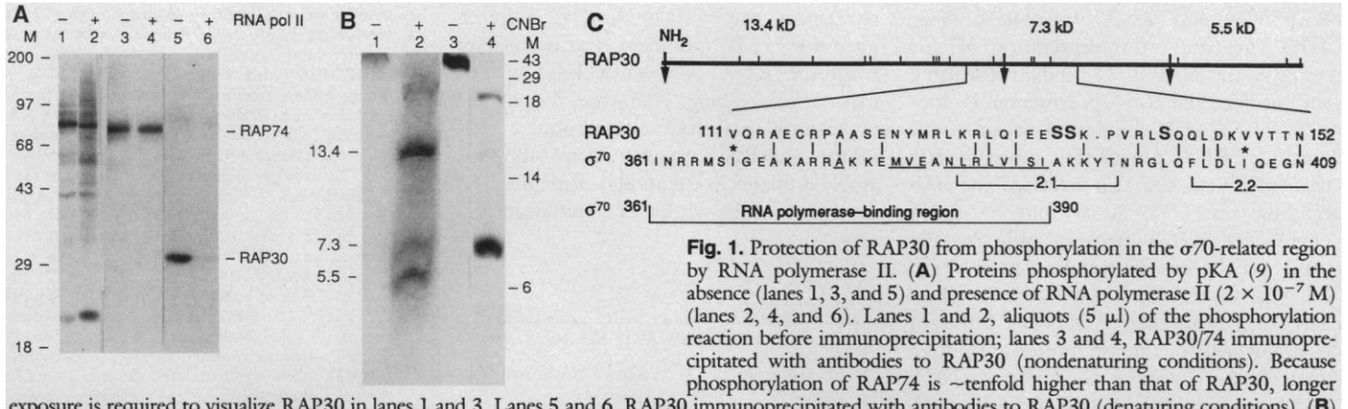

Fig. 1. Protection of RAP30 from phosphorylation in the σ70-related region by RNA polymerase II. (**A**) Proteins phosphorylated by pKA (*9*) in the absence (lanes 1, 3, and 5) and presence of RNA polymerase II (2×10^{-7} M) (lanes 2, 4, and 6). Lanes 1 and 2, aliquots (5 μl) of the phosphorylation reaction before immunoprecipitation; lanes 3 and 4, RAP30/74 immunoprecipitated with antibodies to RAP30 (nondenaturing conditions). Because phosphorylation of RAP74 is ~tenfold higher than that of RAP30, longer exposure is required to visualize RAP30 in lanes 1 and 3. Lanes 5 and 6, RAP30 immunoprecipitated with antibodies to RAP30 (denaturing conditions). (**B**) RAP30 labeled in vivo with [^{3}H]leucine (lanes 1 and 2) and RAP30 labeled with ^{32}P in vitro with pKA (lanes 3 and 4) before (lanes 1 and 3) and after (lanes 2 and 4) treatment with cyanogen bromide. Samples were analyzed by electrophoresis in the presence of SDS and urea (*16*). (**C**) Schematic representation of RAP30 highlighting the region (amino acids 111 to 152) with sequence similarity to σ70 (amino acids 361 to 409). The RNA polymerase–binding region of σ70 (amino acids 361 to 390) (*7*) is shown. Underlined amino acids are conserved among members of the σ factor family (*8*). Methionine residues in RAP30 are denoted by arrows; serine residues by vertical lines in the top diagram. Vertical lines between RAP30 and σ70 show identical amino acids; asterisks show conservative substitutions. Serines potentially phosphorylated by pKA appear in large, bold type. Abbreviations for the amino acid residues are as follows: 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. All fragment sizes are given in kilodaltons. M, molecular-size markers.

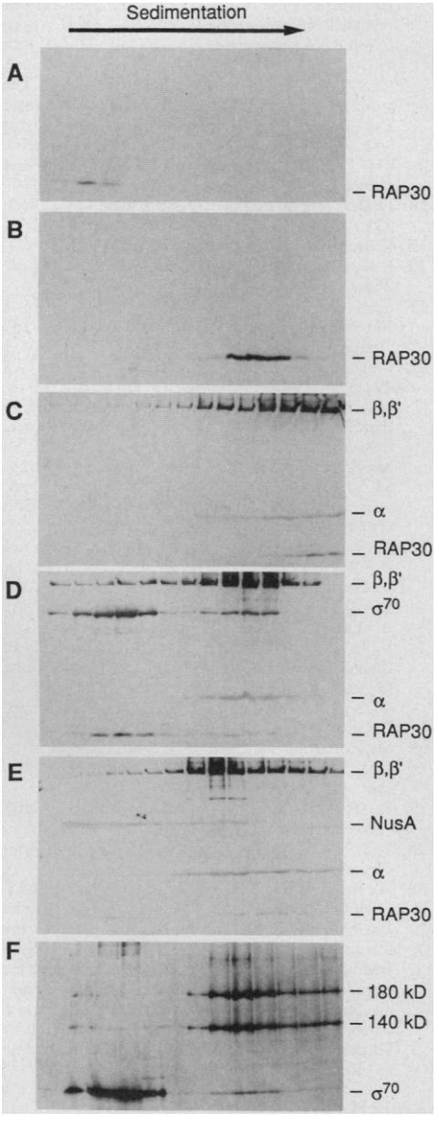

Fig. 2. Sedimentation analysis of RAP30/74 and *E. coli* RNA polymerase. Protein samples were sedimented on glycerol density gradients as described (*17*). Samples applied to gradients contained the following: (**A**) RAP30/74 (1×10^{-9} M); (**B**) RAP30/74 (1×10^{-9} M) and calf thymus RNA polymerase II (4×10^{-7} M); (**C**) RAP30/74 (1×10^{-9} M) and *E. coli* RNA polymerase (4×10^{-7} M); (**D**) RAP30/74 (1×10^{-9} M), *E. coli* RNA polymerase (4×10^{-7} M), and excess σ70 (1.1×10^{-6} M); (**E**) RAP30/74 (1×10^{-9} M), *E. coli* RNA polymerase (4×10^{-7} M), and excess NusA (9×10^{-7} M); and (**F**) σ70 (1.1×10^{-6} M) and calf thymus RNA polymerase II (4×10^{-7} M). Fractions (300 μl) were collected, lyophilized, analyzed on SDS-polyacrylamide gels, and either stained with silver as in (F) or transferred to nitrocellulose and probed with antibodies to RAP30, NusA, and the subunits of the *E. coli* RNA polymerase: β, β', α, and σ70 as in (A through E). The RNA polymerase II was not shown in (B) but cosedimented in the same position as in (F) (180- and 140-kD subunits).

RAP74, which coimmunoprecipitated with RAP30 under nondenaturing conditions (lanes 3 and 4); nor did it affect the phosphorylation of contaminating proteins in the reaction (lanes 1 and 2). This specific protection of RAP30 by RNA polymerase II is consistent with the suggestion (*3, 5*) that it is the RAP30 subunit of RAP30/74 that binds directly to RNA polymerase II and indicates that the pKA phosphorylation site on RAP30 is located in or near the polymerase-binding domain.

Phosphoamino acid analysis (*10*) and two-dimensional tryptic peptide mapping (*11*) showed that RAP30 was phosphorylated by pKA on a serine residue in a single tryptic peptide (*12*). In order to localize this pKA phosphorylation site in RAP30, we used cyanogen bromide (*13, 14*) to hydrolyze RAP30 at methionine residues (Fig. 1B). Cyanogen bromide treatment of RAP30 labeled in vivo with [^{3}H]leucine (*15*) produced three fragments (lane 2) whose sizes, as predicted from the cDNA sequence, were 13.4, 7.3, and 5.5 kD (Fig. 1C). By comparing the electrophoretic mobility (*16*) of the phosphorylated cyanogen bromide fragment to the mobilities of these peptide fragments, we determined that the site of pKA phosphorylation was located in the 7.3-kD peptide (Fig. 1B, lane 4). Therefore, pKA phosphorylated RAP30 on at least one of the serine residues at positions 135, 136, or 142. These serines in RAP30 are located immediately COOH-terminal to the region that is similar to the RNA polymerase–binding domain of σ70 (Fig. 1C) (*7*). Protection of this site from phosphorylation by RNA polymerase II supports the idea that the σ70-related region of RAP30 is a polymerase-binding domain.

We next tested whether human RAP30/74 might bind to the *Escherichia coli* RNA polymerase core enzyme by determining if RAP30/74 cosedimented with *E. coli* RNA polymerase in glycerol-density gradients (*17*). Affinity-purified RAP30/74 sedimented at 5*S* (*3*) (Fig. 2A). However, RAP30/74 sedimented further in the gradient, to the same position as RNA polymerase II (Fig. 2F), when both RAP30/74 and RNA polymerase II were included in the sample (*3, 18*) (Fig. 2B). RAP30/74 also cosedimented with the core component of *E. coli* RNA polymerase under the same conditions (Fig. 2C). The presence of a threefold excess of σ70 over RNA polymerase inhibited cosedimentation of

RAP30/74 and RNA polymerase (Fig. 2D). The simplest interpretation of this result is that RAP30/74 binds to the same site on bacterial RNA polymerase as does σ70. The *E. coli* elongation factor NusA binds to RNA polymerase more weakly than σ70 at a site that overlaps the σ70 binding site (*19*). A twofold excess of NusA over RNA polymerase only partially inhibited the cosedimentation of RAP30/74 with bacterial RNA polymerase (Fig. 2E), suggesting that RAP30/74 binds to *E. coli* RNA polymerase more tightly than does NusA. Therefore, the abilities of σ70 and NusA to displace RAP30/74 from *E. coli* RNA polymerase are directly related to the binding affinities of these factors for RNA polymerase. In the converse experiment, a portion of the σ70 applied to the gradient cosedimented with mammalian RNA polymerase II (Fig. 2F), indicating that σ70 can bind, although weakly, to RNA polymerase II. Gradients with σ70 alone did not show a peak of σ70 sedimenting at the position of RNA polymerase II (*12*).

The approximate dissociation constants for initiation factor–RNA polymerase interactions calculated from the sedimentation experiments (Table 1) indicate that RAP30/74 bound to *E. coli* RNA polymerase and mammalian RNA polymerase II with comparable affinities. Unlike RNA polymerase II, *E. coli* RNA polymerase cannot efficiently protect RAP30 from phosphorylation by pKA (*12*). This may be because the pKA phosphorylation site is located adjacent to, not within, the predicted RNA polymerase–binding site (7) (Fig. 1C), and the structural differences of the *E. coli* enzyme do not permit protection of this region.

Our results show that RAP30/74 and σ70 have functionally similar RNA polymerase–binding sites. Moreover, σ70 and the rat liver transcription factor βγ (*20*), which cross-reacts with antibodies to human RAP30 and RAP74 (*12*), both displace RNA polymerase that is nonspecifically bound to DNA (*21*). This conservation of function between σ70 and RAP30/74 indicates that RAP30/74 may be considered a member of the σ factor family (*8*). Moreover, it is consistent with the sequence similarities of the large subunits of prokaryotic and eukaryotic nuclear RNA polymerases (*22*). However, σ factors recognize bacterial promoter sequences (*23*), whereas RAP30/74 has not been shown to participate in promoter recognition. TFIID (*24*), which recognizes the TATA sequence of eukaryotic promoters, has a region with limited sequence similarity to the σ70 region that recognizes the −10 region of bacterial promoters (*25*), a domain that is separate from the RNA polymerase–binding domain discussed here. Therefore, the regions of bacterial σ factors that recognize promoters and RNA polymerase may be separable domains in eukaryotes distributed to at least two distinct general initiation factors.

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15. We prepared labeled RAP30 by labeling HL-60 cells (1×10^7 cells per milliliter) with [^{3}H]leucine (145 Ci/mmol, 0.5 mCi/ml) (Amersham, Arlington Heights, IL) for 16 hours at 37°C in RPMI-1640 medium (*26*) that lacked leucine and contained dialyzed fetal calf serum (10%). The cells were washed in cold phosphate-buffered saline (*27*) and lysed in 2.0 ml of buffer with Hepes (10 mM), pH 7.9, EDTA (10 mM), EGTA (10 mM), sodium fluoride (5 mM), sodium orthovanadate (5 mM), sodium molybdate (5 mM), sodium pyrophosphate (5 mM), phenylmethylsulfonyl fluoride (1 mM), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (100 μg/ml), *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK) (100 μg/ml), aprotinin (5 μg/ml), leupeptin (2 μg/ml), and pepstatin (2 μg/ml). The chromatin was extracted in NaCl (0.4 M) for 30 min at 0°C. After centrifugation (14,000*g*, 10 min), the supernatant was denatured and RAP30 was immunoprecipitated as described (*9*). RAP30 that was phosphorylated with [γ-^{32}P]ATP and pKA was prepared as described (*9*).
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