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Specificity of the protein kinase activity associated with the hemin-controlled repressor of rabbit reticulocyte

(protein synthesis/translational control/hemin regulation/Met-tRNA$_f$ binding factor)

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ABSTRACT Highly purified preparations of hemin-controlled repressor of rabbit reticulocyte contain a 3':5'-cyclic AMP-independent protein kinase activity that phosphorylates the low-molecular-weight (about 38,000) polypeptide chain of the initiation factor that forms a ternary complex with GTP and Met-tRNA$_f$. These preparations also phosphorylate several polypeptide components of reticulocyte 40S ribosomal subunits. However, no significant levels of phosphorylation are observed when casein, histones, *Artemia salina* 40S ribosomal subunits, or other initiation factor fractions are used as substrates although high levels of phosphorylation are obtained with cruder preparations of the repressor. An antibody to these highly purified preparations of repressor has been obtained from the serum of immunized goats. Preincubation with immune goat IgG results in the neutralization of the inhibitory activity of the repressor, while normal IgG has no effect. Preincubation with immune IgG also abolishes the protein kinase activity responsible for the phosphorylation of the initiation factor and reticulocyte 40S subunits. Histone phosphorylation by crude repressor preparations, on the other hand, is unaffected by preincubation with immune IgG.

Globin synthesis in both rabbit reticulocytes (1, 2) and their cell-free lysates (3, 4) is controlled by the availability of hemin. The cessation of protein synthesis observed in the absence of added hemin is due to a block in initiation of new globin chains (3–7) and appears to involve the depletion of 40S ribosomal subunit·Met-tRNA$_f$ initiation complexes (8, 9). An inhibitory protein, the hemin-controlled repressor (HCR), has been isolated from reticulocyte lysates incubated without hemin (10) which, when added to hemin-supplemented lysates, produced inhibition kinetics similar to those induced by hemin deficiency (11) and promotes the disappearance of 40S·Met-tRNA$_f$ initiation complexes (8, 12).

Hunt and coworkers (13, 14) have reported that millimolar amounts of 3':5'-cyclic AMP (cAMP), 2-aminopurine, or GTP prevent the inhibition of protein synthesis observed in reticulocyte lysates incubated in the absence of hemin. They also observed that ATP counteracted the effect of GTP (14). These results have been confirmed by the work of Ernst *et al.* (15). It has been proposed that the HCR acts as a protein kinase, thus providing a possible explanation for the effect of 2-aminopurine on the inhibition of protein synthesis observed in reticulocyte lysates incubated in the absence of hemin (14). London and coworkers (16) have reported that HCR preparations contain a cAMP-dependent protein kinase that phosphorylates calf thymus histone II. They have suggested that this protein kinase activity may phosphorylate the initiation factor that forms a ternary complex with GTP and Met-tRNA$_f$, IF-E$_2$ according to Staehelin's nomenclature (17). They also suggested that this kinase activity may be responsible for the inhibition observed in hemin-deficient reticulocyte lysates. However, evidence has been obtained recently indicating that the histone kinase present in crude preparations of HCR is distinct from the kinase activity that phosphorylates IF-E$_2$ (P. Farrell, K. Balkow, T. Hunt, and R. J. Jackson; Cambridge, England; personal communication).

In this communication we report that highly purified HCR preparations, while devoid of histone kinase activity, contain a cAMP-independent protein kinase activity that phosphorylates the 38,000-dalton polypeptide chain of IF-E$_2$ and certain proteins associated with reticulocyte 40S ribosomal subunits that have been washed with 0.5 M KCl. Furthermore, goat IgG antibody against HCR neutralizes the above-mentioned kinase activity as well as the inhibitory activity of HCR while not having an effect on the histone kinase activity.

METHODS

Fractionation of Reticulocyte and *Artemia salina* Components. Preparation of rabbit reticulocyte ribosomes has been described (18). *A. salina* ribosomes and their subunits were prepared by the procedure of Zasloff and Ochoa (19) as modified by Kramer *et al.* (20). Subunits of reticulocyte ribosomes were prepared by the procedure of Falvey and Staehelin (21) as modified by Obrig *et al.* (22). Ribosomal subunits, concentrated by centrifugation, were active in protein synthesis.

The hemin-controlled repressor, HCR, from rabbit reticulocytes was purified essentially by the procedure of Gross and Rabinovitz (10) except that the order of the steps involved was altered. The postribosomal supernatant fraction of a 1:1 reticulocyte/water lysate was made 5 mM in *N*-ethylmaleimide, incubated for 15 min at 37°, then made 5 mM in dithioerythritol and incubated at 37° for 10 min. The treated supernatant was then precipitated with ammonium sulfate between the levels of 0 and 50% saturation; the precipitate was fractionated by successive chromatography on DEAE-cellulose, hydroxylapatite, phosphocellulose, DEAE-cellulose, and Sepharose 6B. The resulting HCR preparation gives 50% inhibition of protein synthesis in a reticulocyte lysate at a concentration of 0.4 μg/ml.

The 0.5 M KCl ribosomal wash fraction of rabbit reticulocyte (23) was fractionated on DEAE-cellulose as described (24). Three fractions, designated DEAE$_{100}$, FI, and FII, containing initiation factor activity were obtained. Separation from another initiation factor activity and considerably higher purification of IF-E$_2$ was accomplished by chromatography of FI on phosphocellulose. The resulting IF-E$_2$ preparation promoted the GTP-dependent binding of 1800 pmol of Met-tRNA$_f$ to nitrocellulose filters per mg of protein when assayed as described (24). These preparations of IF-E$_2$ gave three prominent bands and several faint bands on heavily overloaded sodium dodecyl sulfate gels.

Sundkvist and Staehelin (25) reported that highly purified IF-E$_2$ had a specific activity of 3523 pmol of Met-tRNA$_f$ bound

Abbreviations: IF-E, initiation factor from a eukaryotic source; cAMP, 3':5'-cyclic AMP; HCR, hemin-controlled repressor.

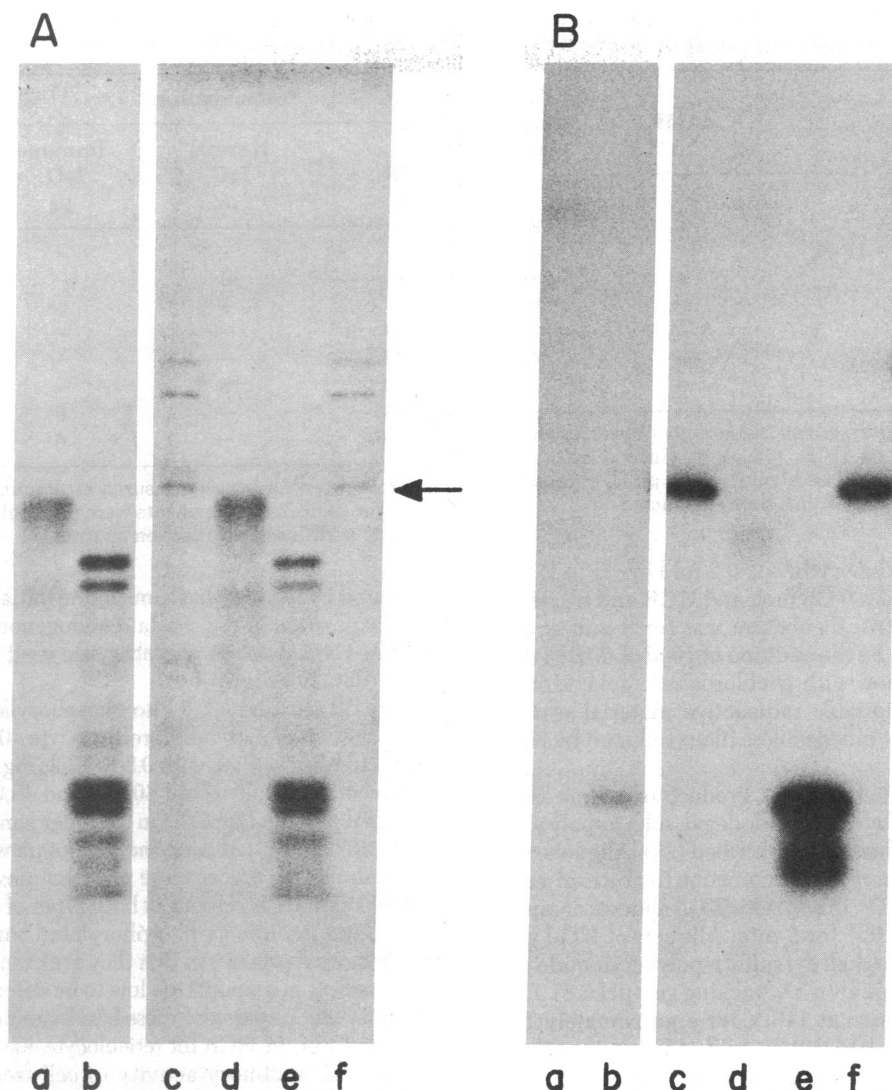

FIG. 1. Polyacrylamide slab gel electrophoresis of protein kinase incubation mixture. Protein kinase assay, slab gel electrophoresis, staining, and autoradiography were performed as described in *Methods*. (A) Stained gel; (B) autoradiograph. (a and d) Casein (6 μg); (b and e) calf thymus histone II (8 μg); (c and f) reticulocyte IF-E_2 (0.8 μg). In addition, a, b, and c contained 0.008 μg of purified HCR, and d, e, and f contained 0.08 μg of partially purified HCR. The position of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (subunit molecular weight = 37,000) is indicated by the arrow.

per mg of protein. IF-E_2 contained three subunits of 32,000, 47,000, and 50,000 daltons (26). Safer *et al.* (27) obtained a specific activity of 550 pmol of Met-tRNA$_f$ bound per mg of homogeneous IF-MP that contained two subunits. Recently (28) they reported that this factor consists of three nonidentical subunits with molecular weights of 38,000, 53,000, and 57,000.

Preparation of Goat IgG against HCR. Two 1- to 2-year-old goats were injected subcutaneously with 4 mg of highly purified HCR in complete Freund's adjuvant. Three additional injections of HCR without Freund's adjuvant were given at 2-week intervals. The goats were bled 1 week after the final injection; the IgG-rich fraction of the plasma was obtained by precipitation with ammonium sulfate between the levels of 0 and 40% saturation and chromatography on DEAE-cellulose (pH 8.0). Normal IgG was prepared from blood obtained prior to immunization.

Protein Synthesis in Reticulocyte Lysates. Rabbit reticulocyte lysates were prepared as described (3). Protein synthesis in lysates was measured as described (24), with minor modifications. Reaction mixtures contained the following in a final volume of 100 μl: 10 mM Tris·HCl (pH 7.5), 90 mM KCl, 1.5 mM $MgCl_2$, 5 mM dithioerythritol, 0.5 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 26 units/ml of creatine phosphokinase, 0.1 mM [^{14}C]leucine (40 Ci/mol), 0.1 mM all other unlabeled amino acids, 5 μg/ml of hemin (bovine, Type I; Sigma Chemical Co., Mo.), and 20 μl of rabbit reticulocyte lysate. Incubation was at 34° for 30 min. Reactions were stopped by diluting 25-μl aliquots with 1.0 ml of a solution containing 0.50 M NaOH and 1.5% H_2O_2. After incubation for 10 min at 37°, the samples were made 5% in trichloroacetic acid and incubated at 90° for 5 min. The precipitate formed was collected on nitrocellulose filters (0.45-μm pore size, Type HAWG, Millipore Corp., Bedford, Mass.) and washed with three 5-ml portions of 5% trichloroacetic acid. Radioactivity was measured by liquid scintillation in 10 ml of toluene counting fluid containing 5.0 g of 2,5-diphenyloxazole per liter of toluene.

Protein Kinase Assay. Incubation mixtures contained the following in a final volume of 100 μl: 20 mM Tris·HCl (pH 7.5),

Table 1. Effect of cAMP on protein kinase activity associated with HCR at different stages of purification

Additions	cAMP (2×10^{-5} M)	^{32}P into protein (pmol)
Partially purified HCR	—	7.3
	+	8.8
Partially purified HCR + IF-E_2	—	18.1
	+	28.5
Purified HCR	—	0
	+	0
Purified HCR + IF-E_2	—	6.8
	+	6.4

Assay conditions were as described in *Methods.* Where indicated, 4.3 μg of partially purified HCR, 0.4 μg of purified HCR, and 10 μg of IF-E_2 were added. A blank value of 1.5 pmol, obtained in samples without IF-E_2 and HCR, has been subtracted.

Table 2. Effect of normal and immune IgG on the inhibitory activity of the hemin-controlled repressor

	Preincubation		
HCR, μg	Normal IgG, μg	Immune IgG, μg	[^{14}C]Leucine incorporated, cpm
—	—	—	34,113
—	6	—	31,847
—	—	6	27,070
0.04	—	—	20,890
0.08	—	—	14,343
0.04	6	—	19,812
0.04	—	6	26,203
0.08	—	6	26,651

Protein synthesis was measured as described in *Methods* except that the indicated components were preincubated at 37° for 20 min prior to addition of the reaction mixture.

1 mM $MgCl_2$, 5 mM dithioerythritol, 0.2 mM [γ-^{32}P]ATP adjusted to an average of 1500 Ci/mol, and HCR and substrates in the indicated amounts. Incubation was for 8 min at 37°. Reactions were stopped by the addition of 10 μl of 0.10 M ATP followed by precipitation with trichloroacetic acid. Hot trichloroacetic acid-precipitable radioactive material was determined by filtration on nitrocellulose filters followed by liquid scintillation counting.

Analysis of ^{32}P-Labeled Proteins. Products of protein kinase assays were analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis as described (29). Aliquots of 20 μl were withdrawn from reacted incubation mixtures after the addition of unlabeled ATP, mixed with 30-μl aliquots of sample buffer, and heated at 100° for 5 min. Aliquots of 20 μl were applied to a 15% sodium dodecyl sulfate-polyacrylamide running gel (pH 8.8) overlaid by a 5% stacking gel (pH 6.8). Electrophoresis was performed at 110 V for approximately 7 hr. Proteins were fixed in 10% acetic acid, 45% methanol and stained with Coomassie brilliant blue. Gels were destained electrophoretically, dried, and put under an x-ray film ("no screen", Kodak) for about 36 hr.

RESULTS

Protein kinase activity associated with HCR

The effect of partially and highly purified HCR on the phosphorylation of casein, histones, and IF-E_2 is shown in Fig. 1. Highly purified HCR prepared as described in *Methods* promotes the phosphorylation of the low-molecular-weight subunit of IF-E_2 (molecular weight, approximately 38,000) while having very little activity with histones (Fig. 1B; b and c). This HCR preparation had a specific activity as defined by Maxwell *et al.* (11) of approximately 25,000. On the other hand, partially purified HCR from the hydroxylapatite step (specific activity of approximately 2500) promotes the efficient phosphorylation of both IF-E_2 and histones (Fig. 1B; e and f). Thus, it appears that the histone kinase present in crude preparations of HCR is a contaminant rather than an activity related to the inhibitory function of the HCR. Neither the highly purified nor the partially purified (hydroxylapatite step) HCR promotes the phosphorylation of casein (Fig. 1B; a and d), even though a casein kinase is present in postribosomal supernatants of rabbit reticulocyte (data not shown). Highly purified HCR does not appear to phosphorylate to any appreciable extent any of the other initiation factor fractions (ref. 24 and *Methods*) available in this laboratory (data not shown). However, these results are obscured by the fact that some of the initiation factor fractions, unlike purified IF-E_2, contain endogenous protein kinase activities. HCR does not phosphorylate itself under the conditions used (data not shown).

Highly purified HCR also phosphorylates several polypeptides associated with rabbit reticulocyte 40S ribosomal subunits that had been washed with 0.5 M KCl (Fig. 2B; d). Two of these (molecular weights about 30,000 and 48,000) appear to be ribosomal proteins (30, 31). On the other hand, ribosomal proteins from 40S subunits of *A. salina* are not phosphorylated (Fig. 2B; b) even though one of these polypeptides, namely, the one of 48,000 daltons, is present in both types of ribosomes (Fig. 2A). The other intensively phosphorylated bands do not appear to be ribosomal proteins in that they are of high molecular weight and present in amounts too low to be detected by staining. The identity and function of these bands are unclear. The apparent specificity of the HCR for reticulocyte 40S subunits may explain its lack of inhibitory activity in cell-free, fractionated, protein-synthesizing systems using *A. salina* ribosomes (24).

The data presented in Table 1 indicate that cAMP stimulates protein kinase activity of partially purified HCR from the hydroxylapatite step of the isolation procedure. No stimulation by cAMP was observed with the highly purified HCR preparation over a concentration range of 10^{-7} M to 10^{-4} M. Thus it appears that the stimulation of kinase activity reported by other workers (16) is an effect on the histone kinase rather than on the protein kinase closely related to HCR activity.

Effect of anti-HCR IgG on inhibitory and protein kinase activities

The effect of normal and immune goat IgG on the inhibitory activity of reticulocyte HCR is shown in Table 2. Six micrograms of immune IgG completely neutralize at least 0.08 μg of purified HCR, while the same amount of normal IgG has no effect on the inhibitory activity of 0.04 μg of HCR.

The histone kinase activity present in the partially purified HCR is not abolished by anti-HCR IgG (Fig. 3; g and h), nor is the small amount of residual histone kinase activity present in highly purified HCR (Fig. 3; a and b). On the other hand, preincubation of either HCR preparation with immune IgG prevents the phosphorylation of IF-E_2 (Fig. 3; d and f). Phosphorylation of reticulocyte proteins associated with the 40S ribosomal subunit is also inhibited by the antibodies (Fig. 3; j). These results indicate that the histone kinase and inhibitory activities observed in crude HCR preparations are distinct

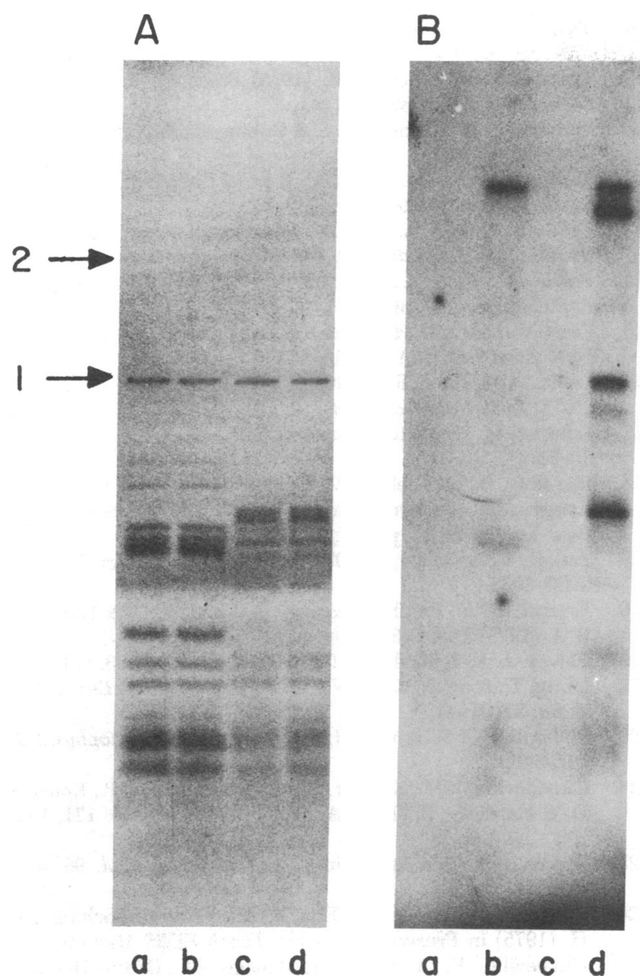

FIG. 2. HCR-dependent phosphorylation of reticulocyte and *A. salina* 40S ribosomal subunits. Protein kinase assay, slab gel electrophoresis, staining, and autoradiography were performed as described in *Methods*. (A) Stained gel; (B) autoradiograph. (a and b) *A. salina* 40S subunits (10 μg); (c and d) reticulocyte 40S subunits (10 μg). In addition, b and d contained 0.008 μg of purified HCR. Arrows indicate the positions of *1*, fumarase (EC 4.2.1.2) (subunit molecular weight about 48,500) and *2*, bovine serum albumin (molecular weight about 68,000).

entities while suggesting a functional relationship between the inhibitory activity of HCR and its IF-E_2-40S reticulocyte subunit kinase activity.

DISCUSSION

London and coworkers (16) have reported a cAMP-dependent protein kinase activity associated with preparations of rabbit reticulocyte HCR which phosphorylates calf thymus histone II. They also observed that HCR preparations from hemin-supplemented lysates displayed little or no inhibitory activity and reduced cAMP dependency in the protein kinase assay. Recently these workers have suggested that the histone kinase associated with the HCR may act by phosphorylating an initiation factor, namely, IF-E_2, thus inactivating it (15).

Here we have demonstrated that the histone kinase activity present in crude preparations of HCR is a contaminant that may be eliminated by further purification and that is not related to the inhibitory activity of the repressor. In addition, we have shown that highly purified HCR preparations do not exhibit cAMP dependency for its kinase activity. The distinct nature of the histone kinase and inhibitory activities was confirmed by the use of antibodies obtained from goats immunized against highly purified HCR. Anti-HCR IgG had no effect on histone phosphorylation by crude HCR preparations while neutralizing the inhibitory activity of the repressor.

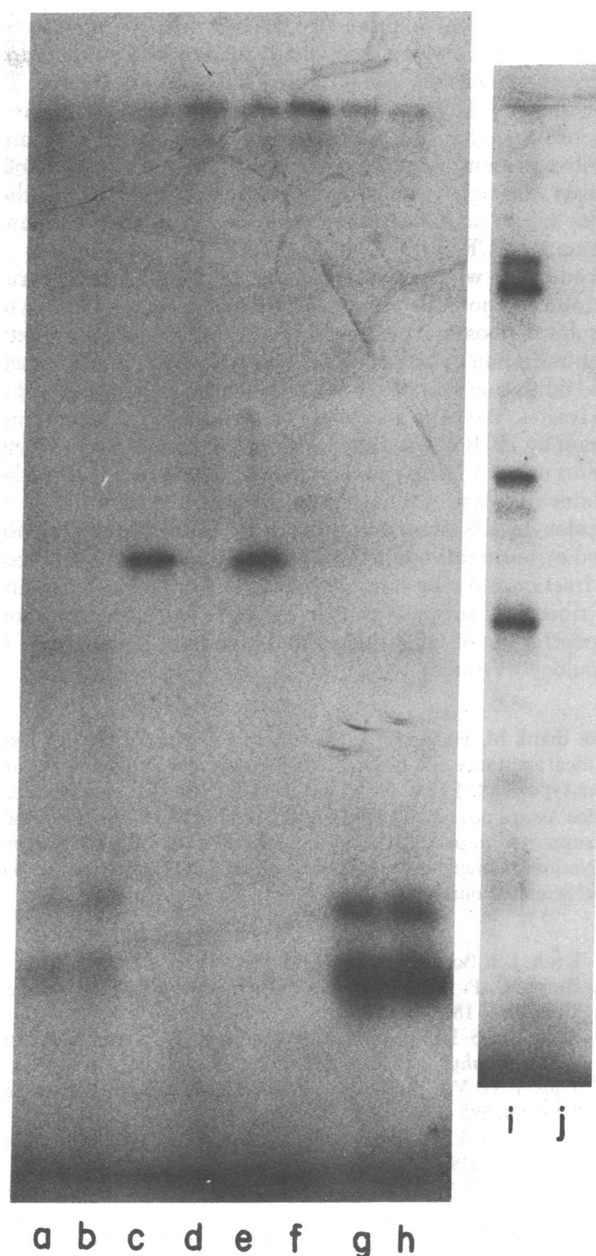

FIG. 3. Effect of immune IgG on HCR-associated protein kinase activity. Protein kinase assay, slab gel electrophoresis, and autoradiography were performed as described in *Methods*. (a and b) Histones + highly purified HCR; (c and d) IF-E_2 + highly purified HCR; (e and f) IF-E_2 + partially purified HCR; (g and h) histones + partially purified HCR; (i and j) reticulocyte 40S ribosomal subunits + highly purified HCR; all of them in the same amounts as in Figs. 1 and 2, respectively. In addition, b, d, f, h, and j contained immune IgG that was preincubated with HCR as described in the legend to Table 2. Only the autoradiograph is shown.

The fact that IF-E_2 stimulates protein synthesis in reticulocyte lysates incubated in the absence of hemin or presence of HCR (16, 32) suggests that the phosphorylation of IF-E_2 by highly purified HCR may be related to the inhibitory mechanism of the repressor. Neutralization of both inhibitory and IF-E_2 kinase activities by anti-HCR IgG provides further, al-

though not conclusive, proof that the two activities are due to the same protein and are functionally related. These results are in agreement with those obtained by P. Farrell, K. Balkow, T. Hunt, and R. J. Jackson (personal communication). They have demonstrated that the inhibitory and IF-E_2 specific kinase activities present in reticulocyte postribosomal supernatants copurify through various chromatographic steps, while the histone kinase activity is a contaminant not related to the inhibition by HCR.

In addition, we have observed that purified HCR preparations also promote the phosphorylation of certain reticulocyte 40S subunit ribosomal proteins. The susceptibility of this activity to neutralization by anti-HCR IgG suggests that it is also related to the inhibition exerted by HCR in hemin-deficient reticulocyte lysates. We have previously reported the resistance to inhibition by HCR of cell-free, fractionated, protein-synthesizing systems using *A. salina* ribosomes (24). These results could be explained in view of the apparent specific phosphorylation of reticulocyte 40S ribosomal proteins by HCR. Recent unpublished experiments confirm this assumption. Globin synthesis in a fractionated system using KCl-washed reticulocyte 40S and 60S ribosomal subunits can be inhibited with the same low concentrations of HCR that exhibit high inhibitory activity in reticulocyte lysates.

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