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The respective roles of the protein kinase and pppA2′ p5′ A2′ p5′ A-activated endonuclease in the inhibition of protein synthesis by double stranded RNA in rabbit reticulocyte lysates

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ABSTRACT

Double-stranded RNA (dsRNA) inhibits protein synthesis in rabbit reticulocyte lysates by activating the synthesis of the endonuclease effector pppA2'p5'A2'p5'A (2-5A) and a protein kinase which phosphorylates the protein synthesis initiation factor eIF-2. Under certain assay conditions, high concentrations of dsRNA are without inhibitory effect in many lysates (high dsRNA "reversible" lysates). In these lysates natural dsRNA at low concentrations stimulated protein kinase activity to a greater extent than did the synthetic dsRNA poly rI.rC. Synthesis of 2-5A was greater when poly rI.rC was used. However, a number of factors, including the salt concentration and messenger RNA used, combine to determine the overall effect of dsRNA on protein synthesis under any given set of experimental conditions.

INTRODUCTION

Double-stranded RNAs inhibit protein synthesis in rabbit reticulocyte lysates and in cell-free systems prepared from a variety of cells pretreated with the antiviral agent interferon (1-4). Two apparently unrelated dsRNA-mediated mechanisms of inhibition have been implicated in the reticulocyte lysate. First, inhibition of initiator Met-$tRNA_f$ binding to 40s ribosomal subunits which has been correlated with phosphorylation of the small subunit of the initiation factor eIF-2 (protein kinase mediated inhibition, 1). Second, degradation of mRNA mediated by the activation of an endonuclease by the oligonucleotide effector pppA2'p5'A2'p5'A (2-5A, 5-9). The inhibition of protein synthesis by very low concentrations of dsRNA (10^{-7} to 10^{-9}g/ml) can, in some lysates under appropriate conditions, be reversed even when well established, by the addition of excess eIF-2 (1). This suggests that the dsRNA-mediated inhibition under these circumstances must act primarily through the protein kinase since the endonucleolytic cleavage of mRNA, once this has

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occurred in response to 2-5A, is unlikely to be reversible. The low dsRNA mediated inhibition can also be prevented from developing by adding the purine analog, 2-aminopurine, or high concentrations (10^{-6} to 10^{-4} g/ml) of dsRNA during the first 8-10 minutes of incubation (1). Here we have examined the respective roles of the protein kinase and endonuclease mediated inhibitions of protein synthesis in response to natural and synthetic dsRNAs in lysates showing this latter type of high dsRNA "reversibility". Natural dsRNAs at low concentrations (10^{-7} to 10^{-9}g/ml) activate only the protein kinase. At higher concentrations the protein kinase is inhibited and the enzyme synthesising 2-5A (2-5A synthetase) is activated. Poly rI.rC preparations activate less protein kinase at low concentrations but significantly more 2-5A synthetase than natural dsRNAs over a wide range of concentrations. Although the nature of the dsRNA is important, therefore, in determining the "reversibility" of inhibition this "reversibility" is also dependent upon the salt (KCl) concentration, the mRNA being translated, the kinetics of amino acid incorporation and the level of 2-5A synthetase in any given lysate.

MATERIALS AND METHODS

All radiochemicals were supplied by the Radiochemical Centre, Amersham, England. Stock solutions of polyinosinic acid; polycytidylic acid (poly rI.rC; sodium salt from Sigma, minimum molecular weight 200,000 or P.L. Biochemicals, $S_{20}w \geq 12$) in 90mM KCl, 10mM Hepes pH 7.6 and dilutions in 10mM Hepes were stored at -20°C. Reovirus dsRNA was extracted from virions with phenol SDS. _Penicillium chrysogenum_ phage dsRNA was a gift from Dr C. Burbidge. The growth and purification of EMC virus and the extraction of unlabelled or [^{3}H]uridine labelled EMC RNA (specific activity, 21000 cpm/μg) have already been described (10). VSV mRNA labelled with [^{3}H] UTP (4 x 10^5 cpm/μg was prepared _in vitro_ and purified by chromatography on oligo (dT)-cellulose (11).

Cell-free systems and assay of 2-5A. Rabbit reticulocyte lysates were prepared and assayed (with KCl added at the concentrations indicated) with or without prior treatment with micrococcal nuclease (12) as described previously (13,14). 2-5A was assayed by the amount required to produce a 50% inhibition of protein synthesis in an EMC RNA-programmed L-cell-free system compared with standard preparations assayed in parallel (10,15,16).

Detection of protein kinase activity. Phosphorylation of exogenous

eIF-2 (20% purified, generously supplied by Dr M.J. Clemens) by the dsRNA dependent protein kinase was monitored by incubating 1μg of eIF-2 with 0.2A_{260} units of 6B ribosomes (prepared by passing a reticulocyte lysate through 6B Sepharose, 1) 1μCi of [γ-^{32}]ATP (specific activity 3000 Ci/mM) 0.1mM ATP, 10mM Tris-HCl pH 7.6, 5mM NaCl, 2mM magnesium acetate and 5% glycerol. After 15 min at 30°C the samples were analysed by SDS-polyacrylamide gel (10%) electrophoresis and autoradiography. Phosphorylation of endogenous eIF-2 was similarly monitored except that eIF-2 was omitted and the concentration of 6B ribosomes increased to 0.9A_{260} units. Detection of endonuclease activity. Lysates were incubated under protein synthesis conditions at 75 or 120mM KCl as indicated in the presence of 0.7μg per 25μl of [^{3}H]EMC or VSV RNA for 60 min at 30°C and samples analysed by SDS polyacrylamide gel (7.5%) electrophoresis and fluorography. Ribosomal and tRNA were included as markers and located by staining with 0.1% toluidene blue, 40% methoxyethanol for 30 min at 37°C then destaining in 30% methoxyethanol.

RESULTS

The high dsRNA "reversibility" phenomenon

Poly rI.rC gave a maximum inhibition of protein synthesis, in the lysates used here, at concentrations of 10^{-7}g/ml. At higher concentrations (10^{-5}g/ml) the inhibition was substantially "reversed" but paradoxically, 2-5A levels peaked (Fig. 1). The absence of detectable 2-5A at lower dsRNA concentrations (10^{-6} to 10^{-8}g/ml) in these systems is a reflection of the relative insensitivity of the assay used and does not reflect the absence of a functional 2-5A system (see below and Fig. 7). We have obtained identical results to those in Fig. 1 with lysates from our own laboratory and from Drs D.H. Levin and R.J. Jackson. As we will show below, it appears that in these systems the high dsRNA "reversible" component of the inhibition is mediated through the protein kinase while the inhibition remaining at high dsRNA (25% at 10^{-5}g/ml, Fig. 1) reflects activation of the 2-5A mediated endonuclease. However, the observation of high dsRNA "reversibility" in a lysate depends upon the assay conditions used. This reflects, in part, the relatively rapid inhibition of protein synthesis resulting from activation of the protein kinase (1) compared to the longer lag in response to 2-5A (6).

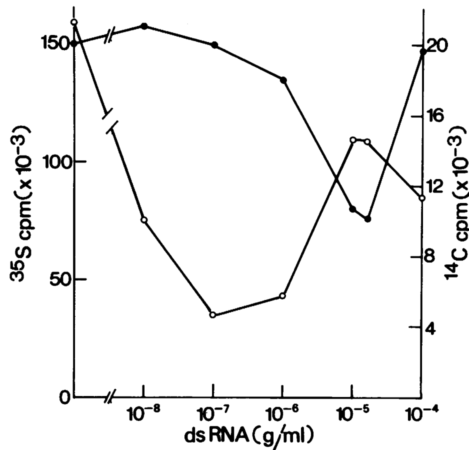

Figure 1. dsRNA concentration dependent inhibition of protein synthesis and formation of 2-5A in the reticulocyte cell-free system. Native lysates without micrococcal nuclease treatment were incubated under protein synthesis conditions and monitored for [^{35}S]methionine incorporation (○ , cpm/5μl) at 60 min in the presence of increasing concentrations of poly rI.rC. 2-5A in these systems was monitored at 60 min: samples were diluted 10 fold, heated at 95°C, centrifuged, and the supernatants assayed at a final dilution of 125 fold in the L-cell-free system where C^{14} amino acid incorporation (cpm/10μl) was monitored after 120 min (●).

Effect of KCl concentration and different mRNAs on high dsRNA "reversibility"

The effect of high (10^{-5}g/ml, ▲) and low (10^{-7}g/ml, ●) dsRNA on the kinetics of protein synthesis at 75mM (Fig. 2A, D) and 120mM (Fig. 2B and c) KCl are shown for a native reticulocyte lysate with endogenous globin messenger RNA (Fig. 2A and B) and for the same lysate pretreated with micrococcal nuclease with EMC RNA as message (Fig. 2C and D). "Reversibility" at high dsRNA was seen with only one of the four sets of conditions used, i.e. with the native lysate at 75mM KCl (Fig. 2A). Under these conditions protein synthesis was essentially over by 20 min. Accordingly, because of the lag involved in its activation, the 2-5A system is likely to have been ineffective and the protein kinase to have been dominant. In contrast, under the three sets of conditions showing no "reversibility", incorporation occurred more slowly over a longer period, inhibition most obviously effected incorporation occurring after 20 min and was likely to be mediated largely through 2-5A. Experiments using the purine analogue, 2-aminopurine, (2AP, an inhibitor of the

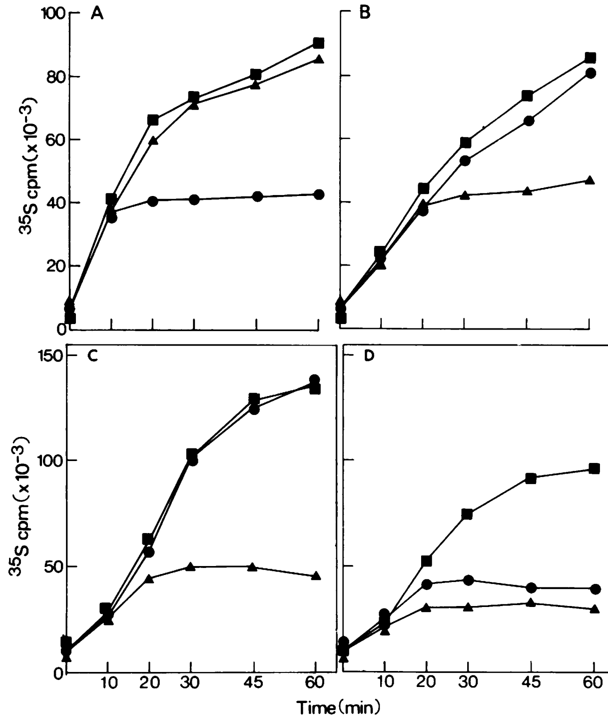

Figure 2. The effect of KCl concentration on the kinetics of protein synthesis in reticulocyte lysates in the presence of dsRNA. Native (A, B) or micrococcal nuclease (C, D) treated lysates were incubated for 60 min at 30°C in the absence (■) or presence of 10^{-7} (●) or 10^{-5} (▲) g/ml of poly rI.rC. [^{35}S]methionine incorporation (cpm/3µl) in response to endogenous globin (A, B) or added EMC (C, D) RNA was at 75mM (A, D) or 120mM (B, C) KCl.

protein kinase but not the 2-5A synthetase, 1, A.G. Hovanessian unpublished results) provided further indirect evidence for this.

Effect of 2-aminopurine

The effect of 2AP on the inhibition of protein synthesis under assay conditions where high dsRNA "reversibility" was or was not observed is shown in Table I. Endogenous globin mRNA translation or that of added EMC RNA, at 75mM KCl was maximally inhibited by low poly rI.rC (Table I, see also Figs. 1 and 2A) but this was only partially "reversed" by 2AP (or high poly rI.rC). This indicates the involvement of both 2-5A and protein kinase in this inhibition. In contrast, the inhibition of translation of EMC RNA or endogenous globin mRNA at 120mM KCl by high or low

TABLE I.

The effect of 2-aminopurine on dsRNA-mediated inhibition of EMC and endogenous globin RNA translation in reticulocyte lysates.

Incubation conditions	[^{35}S]methionine incorporation cpm/10µl		% inhibition	
	2 aminopurine (8mM)			
	-	+	-	+
120mM KCl				
EMC RNA alone	332788	282868	0	0
EMC RNA + 10^{-7}g/ml poly rI.rC	282200	249554	15	12
EMC RNA + 2 x 10^{-5}g/ml poly rI.rC	145268	128230	56	55
Endogenous globin mRNA	164118	140586	0	0
Endogenous globin mRNA + 10^{-7}g/ml poly rI.rC	140556	119288	15	15
Endogenous globin mRNA + 2 x 10^{-5}g/ml poly rI.rC	78888	80624	52	43
75mM KCl				
EMC RNA alone	233786	277222	0	0
EMC RNA + 10^{-7}g/ml poly rI.rC	96214	168580	59	39
EMC RNA + 2 x 10^{-5}g/ml poly rI.rC	139158	134698	40	52
Endogenous globin mRNA	191356	225040	0	0
Endogenous globin mRNA + 10^{-7}g/ml poly rI.rC	68504	146408	64	35
Endogenous globin mRNA + 2 x 10^{-5}g/ml poly rI.rC	110492	121166	43	47

poly rI.rC was relatively unaffected by 2AP and, presumably, is mediated by 2-5A alone. It is clear from this and the evidence presented in Fig. 2 that the observation of high dsRNA "reversibility" is dependent both on the assay conditions used and on the mRNA translated. In addition, the extent of "reversibility" observed also depends on the natμre of the dsRNA employed.

The extent of high dsRNA "reversibility" is dependent upon the nature of the dsRNA

Under conditions favourable to protein kinase mediated inhibition (identical to Fig. 2A) the natural dsRNAs from *P. chrysogenum* and reovirus inhibited protein synthesis at significantly lower concentrations and showed a higher degree of "reversibility" than poly rI.rC (Fig. 3). This suggests that the natural dsRNAs are relatively good activators of the "reversible" protein kinase but relatively poor activators of the

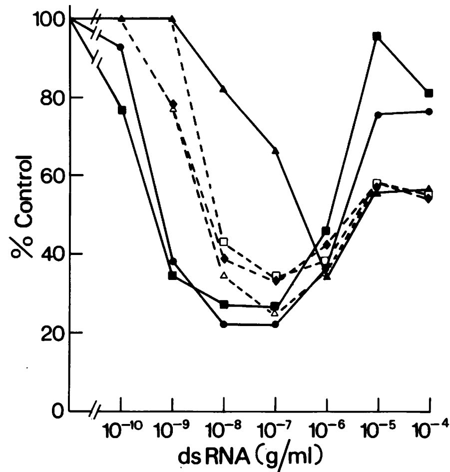

Figure 3. A comparison of protein synthesis inhibition by natural and synthetic dsRNAs. Native lysates were incubated under normal globin synthesis conditions (75mM KCl) in the presence of different concentrations of P. chrysogenum dsRNA (■), reovirus dsRNA (●) poly rI.rC (▲ , Sigma, dilution stored at -20°C), poly rI.rC (△ , Sigma, fresh dilutions ; □ fresh dilution, different batch number) poly rI.rC (◆ , P-L Biochemicals). The results are presented as a percentage of the [^{35}S]methionine incorporation in the absence of dsRNA (153,894 cpm/5µl).

2-5A synthetase while the opposite may be the case for poly rI.rC. Furthermore, the possible importance of the precise structure of the dsRNA in determining its efficacy in the activation of the kinase or synthetase is emphasised by the fact that solutions of poly rI.rC which had been stored in low salt (▲, Fig. 3) were less effective than fresh preparations (△ , □ , ◆) in activating the "reversible" component of the inhibition (Fig. 3).

We have shown that the observation of high dsRNA "reversibility" is dependent on the salt concentration, the mRNA and the nature of the dsRNA. The importance of the mRNA may be simply a reflection of its effect on the kinetics of amino acid incorporation (Fig. 2). The KCl concentration (in addition to its effect on the kinetics of protein synthesis, Fig. 2) and the dsRNA, however, are more likely to effect directly the activation of the synthetase and kinase. This was tested in the experiments outlined below.

Activation of 2-5A synthetase by natural and synthetic dsRNAs and the effect of high KCl

Indirect evidence for a greater activation of 2-5A synthetase by

poly rI.rC than by natural dsRNAs is provided in Fig. 3. This was confirmed in a direct assay under conditions (non-protein synthesising) optimised for 2-5A synthesis in which P. chrysogenum and reovirus dsRNAs were significantly less efficient in activating the synthetase than poly rI.rC (Fig. 4).

The effect of increasing KCl concentration on the accumulation of 2-5A in response to poly rI.rC under protein synthesis conditions in the lysate is shown in Fig. 5. The decrease observed is in accord with the known reversibility of binding of the synthetase to dsRNA at high salt (17) and decreased activity of poly rI.rC Sepharose-bound synthetase at increased KCl (unpublished observations).

Activation of a 2-5A mediated endonuclease by natural and synthetic dsRNAs and the effect of high KCl

Despite the failure to detect 2-5A at high KCl (Fig. 5), sufficient is synthesised (see below, Fig. 7B) under these conditions to activate the endonuclease. This emphasises the inadequacy of the assay of 2-5A by its extraction and dilution ($\geqslant$50 fold) into a second (L) cell-free system. In such an assay the minimum concentration of 2-5A which can be

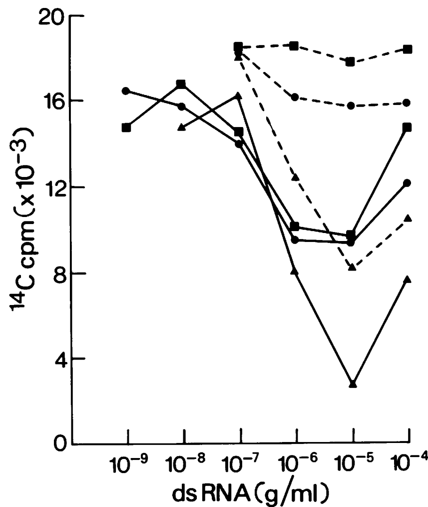

Figure 4. Comparison of activation of 2-5A synthetase by natural dsRNAs and poly rI.rC. Native lysates were incubated with 1mM ATP and the concentrations of poly rI.rC (▲) P. chrysogenum dsRNA (■) or reovirus dsRNA (●) indicated for 60 min at 30°C. 2-5A was assayed in the L-cell-free system as in Fig. 1 at final dilutions of 125 (——) or 1250 (----) fold.

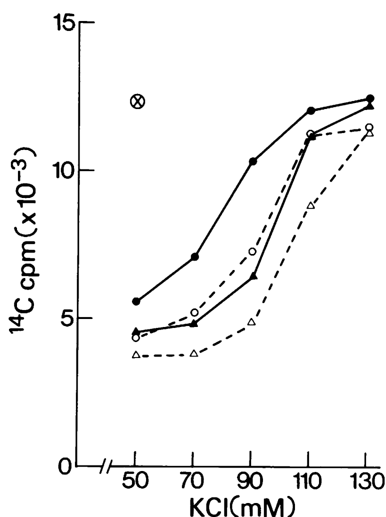

Figure 5. Effect of KCl concentration on the accumulation of 2-5A. Native lysates were incubated at the KCl concentrations indicated under protein synthesis conditions in the presence of 10^{-7} (○ , ○) or 10^{-5} (▲ , △) g/ml of poly rI.rC. Samples taken at 60 min were assayed for 2-5A by inhibition of [^{14}C]amino acid incorporation (ordinate) in the L-cell-free system (see Fig. 1) at final dilutions of 125 (——) or 62.5 (----) fold. The control value (no dsRNA) was 12,400 cpm/10μl (⊗).

detected is determined by the dilution necessary to avoid non-specific inhibition of the L-cell-free system ($\geq$ 50 fold and the concentration ($\geq$ 2 nanomolar) of 2-5A required to produce a specific inhibitory effect in it. The minimum concentration detectable in the lysate in the experiment described in Fig. 5 was, therefore, 100nM. This is in considerable excess over the concentration required both to inhibit protein synthesis ($<$ 1 nanomolar) or produce a detectable activation of nuclease, ($\leq$ 2.4 nanomolar) using an assay with [^{3}H]mRNA as substrate (Fig. 6), in these lysates. The effect of dsRNA and KCl on the activation of endonuclease using this latter type of assay was therefore investigated. Under protein synthesis conditions at 75mM KCl with low concentrations (10^{-8}g/ml) of P. chrysogenum or reovirus dsRNA (tracks 2 and 4, Fig. 7A) where inhibition of protein synthesis was maximal (75 and 76% respectively) there was no more endonucleolytic activity than in control (track 1) or edeine (an initiation inhibitor) treated (track 8) incubations. Poly rI.rC, on the other hand, induced detectable nuclease activity at low concentrations (10^{-8}g/ml, see also ref. 18) even although there was little inhibition

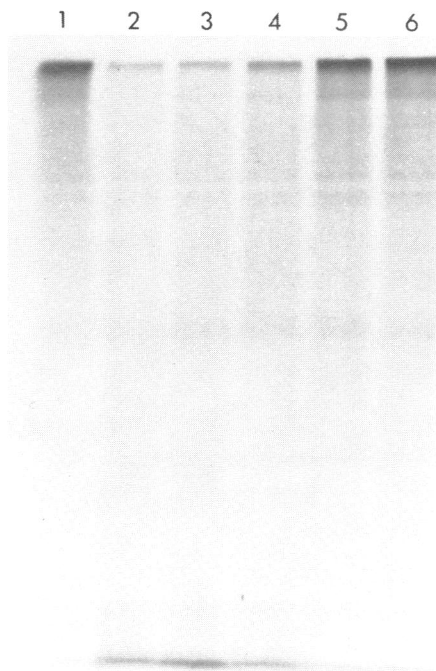

Figure 6. Activation of an endonuclease by 2-5A. Native lysates were incubated with [^{3}H]VSV RNA under protein synthesis condition (75mM KCl) in the presence of different concentrations of 2-5A for 60 min at 30°C. The samples were processed as described in Materials and Methods and a fluorograph of the dried 7.5% SDS polyacrylamide gel is shown. Track 1, control. Tracks 2-6: 24, 8, 2.4, 0.8, 0.24nM 2-5A, respectively.

(19%) of the endogenous globin synthesis which occurs very rapidly and is complete by 20 min under these conditions (Fig. 2A). Significantly more endonuclease was activated at high (10^{-5}g/ml) poly rI.rC (track 7) and natural dsRNA concentrations. These results are in accord with those presented in Fig. 4 and further suggest that under these protein synthesis conditions (native lysate, 75mM KCl) natural dsRNAs are considerably less active than poly rI.rC in activating the 2-5A system.

The effect of high KCl on activation of the endonuclease is shown in Fig. 7B. Despite the failure to detect 2-5A directly (Fig. 4) the activation of significant endonuclease suggests that a reduced but sufficient amount of 2-5A was produced under these conditions for it to be effective in the inhibition observed.

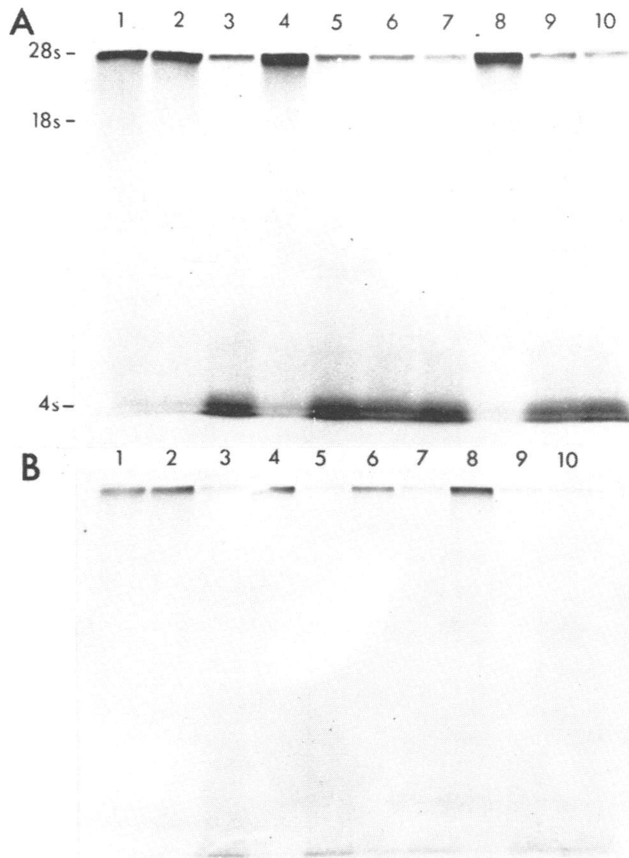

Figure 7. Activation of an endonuclease by dsRNA and the effect of KCl. Native lysates were incubated under protein synthesis conditions in the presence of various inhibitors of protein synthesis and [^{3}H]EMC RNA for 60 min at 30°C. Samples were processed (Methods) and fluorographs of the dried gels are shown. (A). 75mM KCl. (B) 120mM KCl. Track 1, control; tracks 2 and 3, 10^{-8} and 10^{-5}g/ml P. chrysogenum dsRNA, tracks 4 and 5, 10^{-8} and 10^{-5}g/ml reovirus dsRNA; tracks 6 and 7, 10^{-8} and 10^{-5}g/ml poly rI.rC; track 8, 10mM edeine; tracks 9 and 10, 40nM and 80nM 2-5A. The percentage inhibitions of protein synthesis in parallel incubations in tracks 2-10 were 75, 12, 76, 24, 19, 40, 83, 20, 23, respectively. The migration of marker 28s and 18s ribosomal and 4s tRNA is indicated on the left. Tracks 1-10 in (B) are as in (A).

Phosphorylation of eIF-2 mediated by natural and synthetic dsRNAs

In contrast to their reduced effectiveness in activating the 2-5A

synthetase (Figs. 4,7), natural dsRNAs mediated the phosphorylation of eIF-2 at the concentration (10^{-8}g/ml) which gives maximum inhibition of protein synthesis. This phosphorylation was "reversed" at high dsRNA concentrations on both exogenous and endogenous eIF-2 (Figs. 8A and B). The ability of poly rI.rC to mediate the phosphorylation of eIF-2 varied in a manner similar to that demonstrated for inhibition of protein

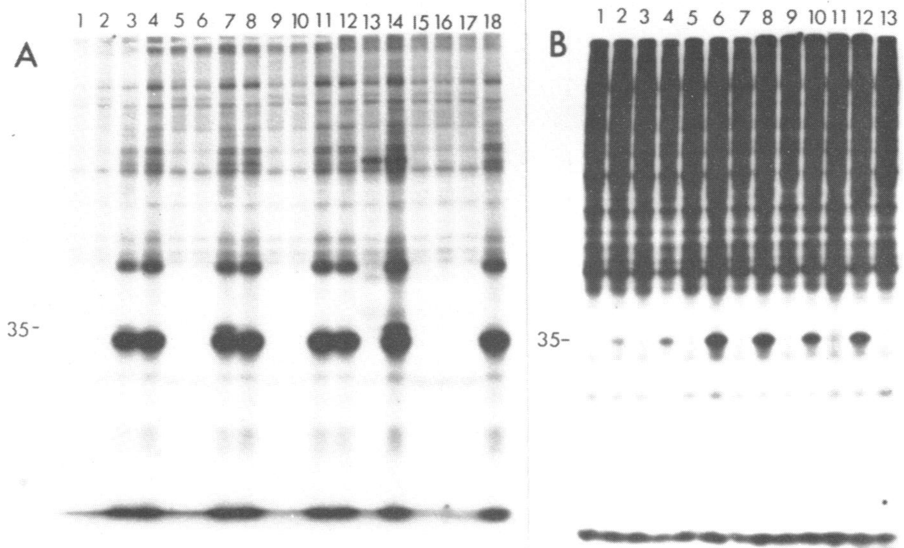

Figure 8. Reversible activation of protein kinase by dsRNA. (A) Phosphorylation of exogenous eIF-2. 0.20 A_{260} units of 6B ribosomes were supplemented with 1μg of eIF-2 (tracks 3, 4, 7, 8, 11, 12, 14, 18 or left unsupplemented (tracks 1, 2, 5, 6, 9, 10, 13, 15-17). Incubations were with [γ-^{32}]ATP (Methods) for 15 min at 30°C in the presence of 10^{-8} (1, 3) or 10^{-5} (2, 4) g/ml P. chrysogenum dsRNA, 10^{-8} (5, 7) or 10^{-5} (6, 8) g/ml reovirus dsRNA, 10^{-8} (9, 11) or 10^{-5} (10, 12) g/ml poly rI.rC, haemin controlled repressor, a protein synthesis inhibitor known to phosphorylate eIF-2; reference 1 (13, 14), H_2O (15, 16), 40nM 2-5A (17, 18). An autoradiograph of the gel is presented. The 35K subunit of eIF-2 is indicated on the left. The heavily phosphorylated band running just below this is a contaminant of the partially purified eIF-2. (B) Endogenous eIF-2 phosphorylation. 0.90A_{260} units of 6B ribosomes were incubated with [γ-^{32}]ATP (Methods) in the presence of H_2O (track 1), 10^{-8} (2) and 10^{-5} (3) g/ml poly rI.rC (Sigma, dilutions stored at -20°C), 10^{-8} (4) and 10^{-5} (5) g/ml poly rI.rC Sigma, fresh solutions), 10^{-8} (6) and 10^{-5} (7) g/ml poly rI.rC (Sigma, fresh dilutions different batch number, 10^{-8} (8) and 10^{-5} (9) g/ml poly rI.rC (P-L Biochemicals), 10^{-8} (10) and 10^{-5} (11) g/ml reovirus dsRNA, 10^{-8} (12) and 10^{-5} (13) g/ml P. chrysogenum dsRNA.

synthesis (Fig. 3). Freshly prepared solutions of poly rI.rC gave "reversible" phosphorylation patterns similar to those shown by natural dsRNAs (Fig. 8B). Preparations stored in low salt, on the other hand, showed reduced activation of protein kinase activity thus providing more direct evidence for the importance of the integrity of the dsRNA in this activation.

It was tentatively concluded from the data presented in Table I and Fig. 2 that the protein kinase did not play a role in dsRNA mediated inhibition at high (120mM) KCl concentrations since no reversible inhibition was demonstrated. This was supported by eIF-2 phosphorylation experiments, performed as in Fig. 8A but at 120mM KCl, which confirmed a reduction in activity under these conditions (data not shown).

Variation in different lysates

High dsRNA "reversibility" is known to vary in different lysates. In accord with this and our interpretation of the results presented above, the levels of 2-5A synthetase in a non-"reversible" lysate were higher than in "reversible" lysates (Table II). Although other factors such as an enhanced level of activatible nuclease or reduced level of protein kinase cannot be excluded it may be that the level of 2-5A synthetase, by

TABLE II.

Comparison of 2-5A synthetase activity in non-"reversible" and "reversible" lysates. (1)

Final dilution of 2-5A in L-cell-free system	Lysate (2) "reversible"	non-"reversible" (3)
	% inhibition in L-cell-free system	
1×10^2	43	78
3×10^2	42	77
1×10^3	3	60
3×10^3	0	47

(1) This variation in 2-5A synthetase activity has been observed in several lysates, although results from only two are presented.

(2) The 2-5A synthetase in each lysate was bound to poly rI.rC paper and incubated with ATP overnight at 30°C (G.R. Stark and I.M.K., in preparation). 2-5A in the supernatants was estimated as in Fig. 1.

(3) From Dr M.J. Clemens.

governing the rate at which 2-5A accumulates, ultimately limits the degree of high dsRNA "reversibility" in any given lysate.

DISCUSSION

The phenomenon of high dsRNA "reversibility" is dependent upon the salt concentration, mRNA and nature of the dsRNA. The effect of the mRNA is likely to be mainly on the kinetics of protein synthesis. However, it remains possible from the data presented in Figs. 2A and C that, in addition, EMC RNA may be more sensitive to 2-5A mediated endonuclease activity than globin mRNA (9,19). Whether this merely reflects an increased endonuclease target size offered by EMC RNA on the one hand, or greater ribosomal protection of globin mRNA on the other, remains to be determined.

The effect of salt is complex. It reduces the effectiveness of low concentrations of dsRNA to activate both the kinase and synthetase (unpublished data and Fig. 5). Nevertheless, even at the high salt concentration optimal for EMC translation (120mM) in the presence of high dsRNA, sufficient 2-5A is activated for the endonuclease to be detected. By increasing the duration of protein synthesis (compare Figs. 2A and B) the high salt may also allow sufficient time for the endonuclease to take effect. Inhibition of protein synthesis by dsRNA at high salt is, therefore, largely 2-5A mediated. Although we have no direct proof of the reduced level of protein kinase activity in the presence of high KCl under complete protein synthesis conditions, the insensitivity of the inhibition to reversal by 2AP (Table I) and the results contained in our original report concerning the involvement of the 2-5A system in reticulocyte lysates (Fig. 10 of 20) would be in accord with this. In contrast, at low salt with endogenous globin mRNA and natural dsRNA the predominant inhibitory (high dsRNA "reversible") effect is through the kinase (Fig. 8) and no detectable nuclease is activated under these conditions (Fig. 7A). The results obtained with poly rI.rC at 75mM KCl with either EMC RNA (Fig. 2D) or endogenous globin as messenger (Fig. 2A) lie between these two extremes, there being good evidence for involvement of both the kinase (Table I; Fig. 3 - high dsRNA "reversible" component; Fig. 8B) and the 2-5A system (Table I; Fig. 3 - residual inhibition at high dsRNA; Fig. 7A).

As far as the 2-5A system is concerned there is some evidence that activation of the 2-5A synthetase may also be "reversible", although

only at significantly higher concentrations of dsRNA (10^{-4}g/ml, Fig. 1). This was noted previously but has not been analysed further (20). Of more immediate interest is the fact that maximum endonuclease activity in these lysates may be reached with as little as 8nM 2-5A. Thus at concentrations of 2-5A in excess of this value it is the level of activatable endonuclease (or an unidentified component of the endonuclease activation system) which becomes limiting and any variation in its level the potential limiting factor in determining the overall effect of dsRNA.

Under any given set of conditions the ability to inhibit protein synthesis is dependent both on the nature and concentration of the dsRNA used (Fig. 3). The natural dsRNAs appear to be better than poly rI.rC in activating the kinase, whereas the reverse seems to be the case for the 2-5A synthetase (Figs. 3,4, 7 and 8). It is possible that this reflects a difference in the detailed structure of the as yet unidentified "natural" activator(s) of these enzymes in the intact cell but this remains to be established. Differential inhibitory effects of natural and synthetic dsRNAs on protein synthesis in reticulocyte lysates have recently been described by Content et al. (21). They found poly rI.rC to be a less efficient inhibitor of protein synthesis than natural dsRNAs but did not investigate the mechanisms of inhibition. Here we have demonstrated that dsRNA mediated inhibition of reticulocyte protein synthesis is the result of a complex combination of the activities of a protein kinase and a 2-5A activated endonuclease. Despite this complexity, however, these systems can be separately analysed and characterised provided the appropriate set of conditions is established.

ACKNOWLEDGEMENT

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Note Added in Proof. The influence of salt concentration on protein kinase mediated inhibition of protein synthesis has recently been discussed by Baglioni et al. (22).

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