

## Binding of mouse interferon to polynucleotides

[purification/poly(I), poly(U)/polynucleotide binding site/blue dextran-Sephadex/affinity chromatography]

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**ABSTRACT** The polynucleotides poly(I) and poly(U), and to a lesser extent poly(G), are capable of desorbing mouse interferon from blue dextran-Sephadex columns, whereas poly(A) and poly(C) are without effect. When covalently bound to agarose, poly(I), poly(U), and also poly(A) act as potent ligands for purification of interferon by affinity chromatography. Furthermore, poly(I) and poly(U) confer a significant degree of protection to interferon against thermal denaturation. Taken together, these observations point to a direct interaction of interferon with these polymers and suggest that interferon molecules have a polynucleotide attachment site. Possible implications for the concept of interferon induction are discussed.

The strong affinity of interferon molecules for the dye Cibacron blue F3GA has been recently reported, independently, from three laboratories (1-3). A practical application of this observation is the purification of interferon by affinity chromatography on blue dextran-Sephadex (1, 2). According to Thompson *et al.* (4), many proteins binding to Cibacron blue F3GA have a supersecondary structure called the "dinucleotide fold" and recognize the chromophore because of its structural similarity to nucleotide cofactors; specific displacement of these proteins from affinity columns is achieved by low concentrations of their specific nucleotide effectors. A few proteins, however, although not possessing the dinucleotide fold, bind to Cibacron blue and require high salt concentrations for elution.

At present, nothing is known concerning the amino acid sequence or the spatial configuration of interferon molecules. We attempted to desorb mouse C-243 cell interferon from a blue dextran-Sephadex column by using various nucleoside phosphates; none of them was able to displace interferon to a significant degree, which suggests that interferon does not have the dinucleotide fold. The possibility that interferon would have other nucleotide phosphate binding sites was envisaged. To test this hypothesis, several single- and double-stranded synthetic ribopolynucleotides were tested for their ability to compete with Cibacron blue F3GA for interferon binding. Some of these compounds were indeed found to be capable of desorbing interferon from blue dextran-Sephadex columns. Results concerning this interaction of mouse interferon with polynucleotides are given in this paper.

### MATERIALS AND METHODS

Blue dextran-Sephadex columns were prepared by coupling blue dextran 2000 to CNBr-activated Sephadex-6B (Pharmacia, Uppsala, Sweden) as described (1). The columns were run at room temperature and, when not in use, were kept at 4° with 0.02% sodium azide in the equilibration buffer.

Lyophilized preparations of synthetic ribopolynucleotides

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were either made in our laboratory or were obtained commercially. Polynucleotides were synthesized by Sephadex-bound polynucleotide phosphorylase as described (5) except poly(G) which was made by the soluble enzyme (6). Poly(I) poly(U), poly(I)-poly(C), and poly(A)-poly(U) were obtained from Laboratoires Choay, Paris, France. The  $s_{20,w}$  value for all polynucleotides used was greater than 5; for poly(I) its range was 5-8 and for poly(U), from 5 to 7.5.

Poly(A) and poly(I) were attached to CNBr-activated agarose through the 5'-phosphate end by the method of Wagner *et al.* (7). Another batch of poly(I) was bound to "hydrazide" agarose, through the oxidized 3' end, by using the technique of Roberson and Davidson (8). Poly(U)-Sephadex 4B was purchased from Pharmacia; in this preparation, the polynucleotide is attached to agarose through multiple points. Columns of polynucleotide-agarose were run at room temperature but otherwise were kept in the cold as described above for blue dextran-Sephadex columns.

Interferon titrations were performed by using a microtiter assay in L cells (0.2 ml of medium per well) with vesicular stomatitis virus (Indiana strain) as challenge virus. One unit corresponds to the minimal amount of interferon capable of conferring protection to 50% of the cells. One microtiter unit per 0.2 ml corresponds to 10 reference units/ml. All titers are expressed in reference units.

Interferon used in this work was made in mouse C-243 cells induced with Newcastle disease virus according to Tovey *et al.* (9). This preparation contained  $1.3 \times 10^6$  units/ml; its protein content was 100  $\mu\text{g}/\text{ml}$ ; and it had a specific activity of  $1.3 \times 10^7$  reference units/mg of protein.

### RESULTS

#### Desorption by polynucleotides of mouse interferon bound to blue dextran-Sephadex

Several polynucleotides were examined for their capacity to desorb interferon from blue dextran-Sephadex columns. As reported (1), when adsorbed onto this type of ligand, a crude preparation of tissue culture interferon is cleaned of >90% of contaminant proteins but the total interferon activity is retained. Results given in Table 1 show that part of the interferon bound to blue dextran can be eluted by 2 bed volumes of a solution of polynucleotides at a concentration of 100  $\mu\text{g}/\text{ml}$  in 10 mM Tris-HCl, pH 7.5. The degree of desorption obtained depended on the nature of the polynucleotide. Poly(I) and poly(U) competed efficiently with blue dextran for interferon binding, because they displaced 80% and 40%, respectively, of the interferon input; poly(G) was less active but still desorbed 17%. The two other single-stranded RNAs tested, poly(A) and poly(C), did not displace interferon significantly. Besides nucleotide configuration, the length of the chain seems to play a role, because oligo(U), in a preparation consisting of a mixture of three- to eight-subunit-long polymers, did not desorb inter-

Table 1. Desorption by RNA polymers of mouse interferon bound to blue dextran-Sephadex

| RNA polymer     | Interferon desorbed, % |
|-----------------|------------------------|
| Poly(A)         | 2                      |
| Poly(C)         | 8                      |
| Poly(G)         | 17                     |
| Poly(I)         | 80, 86*                |
| Poly(U)         | 34, 43*                |
| Oligo(U)†       | 0                      |
| Poly(U,C)       | 1                      |
| Poly(A)-poly(U) | 1                      |
| Poly(I)-poly(C) | 5                      |

Blue dextran-Sephadex columns with bed volumes of 1.5–4 ml were equilibrated with 10 mM Tris-HCl pH 7.5. The interferon preparation was dialyzed in the same buffer. Samples applied were 3–8 ml, which corresponds to  $3.9 \times 10^6$  to  $1 \times 10^7$  units. After the washing step, 2 bed volumes of a given polynucleotide solution, at a concentration of 100  $\mu\text{g/ml}$ , was applied. After another washing step, elution of the interferon remaining in the column was achieved by increasing the ionic strength of the buffer (1 M NaCl). Eluant fractions, calculated to represent one-third of the bed volume, were individually assayed for interferon activity. Shown are the respective percentages of desorption obtained with each polynucleotide tested. Values have been corrected so that percentage desorption due to RNA + percentage of final desorption at high molarity equals 100. Total recovery varied from 70 to 150%, which corresponds to 100% considering the accuracy of the microtest.

\* Results of two experiments.

† Mixture containing polymers of three to eight subunits.

feron at all. In addition, neither the single-stranded copolymer poly(U,C) nor the double-stranded RNAs poly(I)-poly(C) and poly(A)-poly(U) were able to displace interferon.

The fact that only a few polynucleotides were able to compete with blue dextran for interferon binding suggested that the interaction did not depend on a mere effect of charge due to the polyanionic chain. Moreover, oligo-uridylic acid at a concentration of 100  $\mu\text{g/ml}$  [i.e., at a molar concentration 20 to 100 times higher than that of poly(U)] did not displace any interferon from the dye ligand.

The relationship between polynucleotide concentration and amount of interferon displaced was evaluated as follows. Interferon was applied to a column of blue dextran-Sephadex, and the degree of desorption with increasing concentrations of poly(I) and poly(U) was determined. Fig. 1 presents the relative cumulative percentage desorption obtained at a given RNA

Table 2. Binding of mouse interferon to polynucleotides immobilized on agarose

| Poly(A) | Poly(I)            | Poly(U) |
|---------|--------------------|---------|
| 98      | 86 I <sup>3'</sup> | 100     |
|         | 95 I <sup>3'</sup> |         |
|         | 97 I <sup>5'</sup> |         |

Each column contained 2 ml of gel equilibrated in 10 mM Tris-HCl, pH 7.5. The interferon preparation had been dialyzed in the same buffer, and  $1.3 \times 10^7$  units was applied per experiment. After a washing step with 6 ml of the Tris buffer, desorption was carried out by adding 1 M NaCl to the eluant. Fractions (2 ml) were collected, and activity was measured in each of them. The combined total of interferon from the flow-through and from the desorption fractions was considered to be 100%, the absolute value being in all cases close to the theoretical input. Each value given in the table represents the percentage of interferon input retained on the column and subsequently desorbed by increasing the ionic strength of the eluant. I<sup>3'</sup>, Poly(I) chain attached to the agarose by the 3' end; I<sup>5'</sup>, poly(I) attached by the 5'-phosphate end.

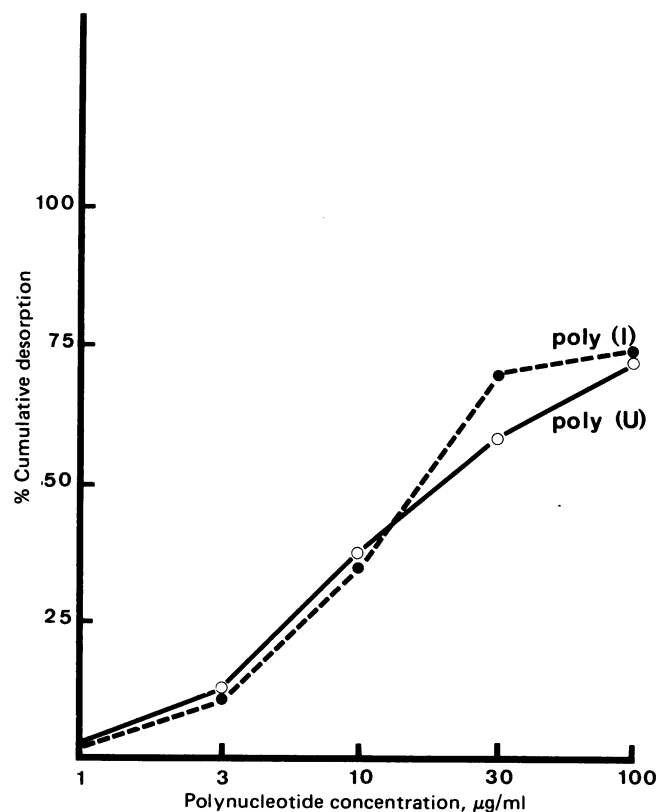


FIG. 1. For both experiments,  $1.5 \times 10^7$  units of interferon was applied to a blue dextran-Sephadex column with a bed volume of 2 ml. After the washing step, desorption was carried out as follows: 4 ml (2 bed volumes) of increasing concentrations of the polynucleotide (1, 3, 10, 30, and 100  $\mu\text{g/ml}$ ) in 10 mM Tris-HCl, pH 7.5, were applied successively to the column. The column was then rinsed and the remaining interferon was desorbed with 1 M NaCl in Tris-HCl buffer. Fractions (2 ml) were collected and interferon was determined in each fraction. The amount of interferon desorbed at each step corresponds to the recovery in two tubes. Values are given in percentage of total relative cumulative desorption.

polymer concentration. Poly(I) at 30  $\mu\text{g/ml}$  was capable of desorbing about 80% of the interferon; no significant increase of desorption was achieved at 100  $\mu\text{g/ml}$ . The results obtained with poly(U) show that increasing concentrations up to 100  $\mu\text{g/ml}$  increased the total amount of interferon desorbed. Yet, the cumulative amount of interferon desorbed with poly(U) (73%) exceeded that obtained with only 2 bed volumes (34–43% as shown in Table 1), which indicates that prolonging the elution at 30–100  $\mu\text{g/ml}$  increases the amount of interferon displaced. This suggests that the protein has a higher affinity for poly(I) than for poly(U). When a similar type of experiment was carried out with poly(A) at increasing concentrations from 100 to 500  $\mu\text{g/ml}$  no interferon was displaced (results not shown).

#### Binding of mouse interferon to immobilized polynucleotides

To ascertain that the displacement of interferon from blue dextran-Sephadex nucleotides resulted from a direct interaction of the protein with the polynucleotide, binding of interferon to polynucleotide-agarose columns was assayed. Crude interferon preparations, dialyzed against 10 mM Tris buffer at pH 7.5, were applied to columns of either poly(A), poly(I), or poly(U) under the conditions described in Table 2 and illustrated in Fig. 2. Practically all the interferon activity was retained by each of these columns, whereas the bulk of the protein was in the flow-through fraction. The total interferon

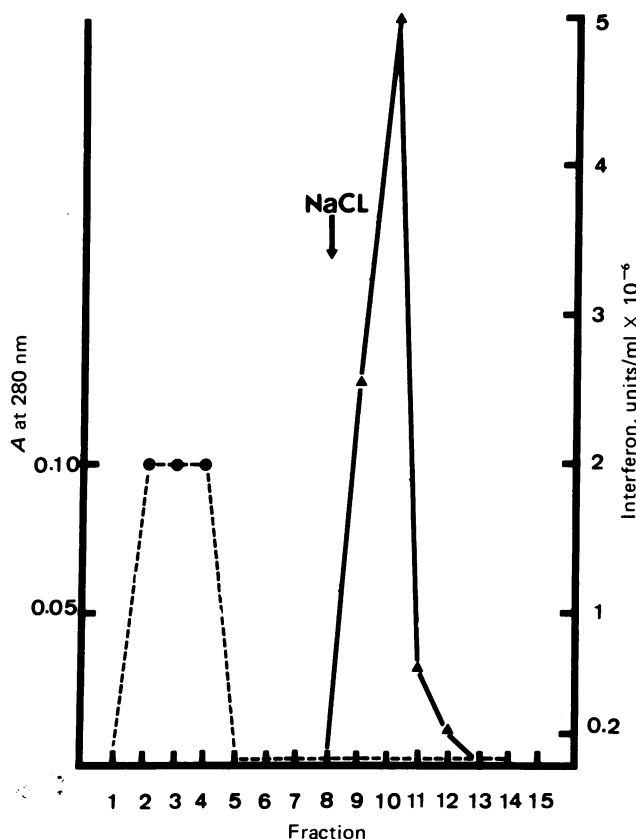


FIG. 2. A total of  $1.3 \times 10^7$  units of interferon was applied to the column (fractions 1–4). After the washing step with 10 mM Tris-HCl buffer (fractions 5–7), the interferon retained on the column was desorbed by adding 10 mM Tris-HCl/1 M NaCl buffer (fractions 8–13). The volume of the fractions was 2 ml. The interferon titer of the peak fraction was  $5 \times 10^6$  units/ml and its protein content was 2.5  $\mu\text{g}/\text{ml}$ . Hence, the specific activity of the peak fraction was  $2 \times 10^9$  units/mg of protein, which corresponds to a 150-fold purification of the starting material. ●—●, Absorbance at 280 nm; ▲—▲, interferon titer.

applied could be recovered by increasing the ionic strength of the solvent (1 M NaCl). Worthwhile noticing are the results with poly(A)-agarose: total binding of interferon was obtained although, at concentrations up to 500  $\mu\text{g}/\text{ml}$ , poly(A) could not displace interferon from the blue dextran-Sephadex gel. Thus, the fact that some RNA polymers do not displace interferon from blue dextran-Sephadex column does not exclude a possibility of binding of interferon to those polyribonucleotides under different conditions.

#### Thermal inactivation curves of mouse interferon in the presence of polynucleotides

Additional evidence for interaction of interferon molecules with polynucleotide chains was obtained by establishing thermal inactivation curves of interferon in the presence of several single- and double-stranded RNA homopolymers. At 60° a crude interferon preparation had a half-life of 2.5 min and retained less than 5% of its activity after 15 min (Fig. 3). When RNA polymers were present at 10  $\mu\text{g}/\text{ml}$ , part of the interferon activity was retained, the amount depending on the polynucleotide. The strongest protective effect was obtained with poly(I) and poly(U), the two polymers capable of desorbing interferon from blue dextran-Sephadex; with these, there was no loss of activity after 30 min and 50% of the initial activity was still present after 60 min. Protection conferred by poly(C)

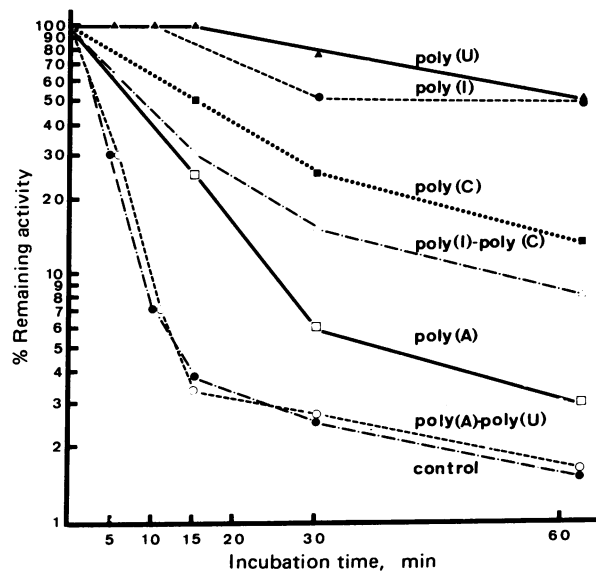


FIG. 3. Aliquots (0.3 ml) containing  $3.9 \times 10^5$  units of interferon in 10 mM Tris buffer at pH 7.5 were incubated at 60° in a water bath. Concentration of the various polynucleotides was 10  $\mu\text{g}/\text{ml}$  (3  $\mu\text{g}$  per aliquot). At the indicated times, 20- $\mu\text{l}$  aliquots were removed, diluted 1:100 in tissue culture medium containing 3% calf serum, and immediately cooled in an ice bath. Interferon titrations were carried out immediately at the end of the incubation.

or by the double-stranded RNA poly(I)-poly(C) averaged 25% after 30 min and 10% after 1 hr. Poly(A) conferred a small degree of protection up to 15 min. The double-stranded poly(A)-poly(U) had no effect.

#### DISCUSSION

The observation that poly(I), poly(U), and to a lesser degree poly(C) were capable of desorbing interferon from blue dextran-Sephadex columns strongly suggested that the RNA polynucleotides were competing with Cibacron blue F3GA for interferon binding. The displacement of the protein from the dye complex is not due to a polynucleotide-dye interaction but is a result of a direct interaction between the protein and the polymer. This statement is based on two lines of evidence. First, interferon was quantitatively retained on a poly(I)-Sephadex or poly(U)-Sephadex column, and it was eluted from the ligand by a high salt solution. Second, the polynucleotides protected interferon from thermal inactivation. However, such interferon-polynucleotide complex formation seems to require a certain structural involvement depending on the nature and the physical properties of the polymers. Indeed, among the polynucleotides being assayed including the five usual homopolynucleotides, the copolymer poly(U,C), the two double-stranded polymers, and oligo(U), only poly(I) and poly(U) efficiently displaced interferon from the dye ligand and equally protected the protein from thermal inactivation.

Except for the keto group and the adjacent NH group on the base, there is nothing suggesting more analogy between poly(I) and poly(U) than among the other polynucleotides tested. At the ionic strength and the pH used, poly(I) (10) and poly(U) (11) assume a single-stranded structure although, at room temperature, poly(U) has minimal stacking. However, the binding of interferon to poly(I) and poly(U) might be mediated by a discrete conformation. This idea is strengthened by our finding that interferon can also bind to some purified specific tRNAs but not to others (12). Somewhat surprising, however, were the results with poly(A): this polynucleotide was totally unable to

desorb interferon from blue dextran, yet all the interferon activity was retained on the poly(A)-Sephadex column. Thus, inability of a given polynucleotide to compete with the blue chromophore for interferon binding does not exclude the possibility of weaker binding forces between this RNA and interferon.

Although the nature of the interferon-polynucleotide interaction is not known, the results obtained preclude a simple charge effect. In addition, they point to a possible existence of a polynucleotide binding site on the interferon molecule. This hypothesis is based on the direct interaction between the protein and the polynucleotide as well as on the binding to blue dextran-Sephadex. It has been found that the blue dextran moiety of the affinity column can recognize a polynucleotide binding site, distinct from the nucleotide binding site, on some proteins known to be specifically bound to the dye. This conclusion is drawn from a study on the interaction of *Escherichia coli* polynucleotide phosphorylase with the blue dextran-Sephadex (unpublished data). The polynucleotide binding site is not restricted to this particular type of mouse interferon because interaction with polynucleotides has also been observed with brain, serum, and immune mouse interferon (unpublished data).

Whether interaction of interferon with polynucleotides has physiological significance remains to be determined. Both poly(I) and poly(U), for which interferon has a strong binding avidity, compose one chain of the double-stranded interferon inducers poly(I)-poly(C) and poly(A)-poly(U), and we believe that it is more than a coincidence that an inducible protein such as interferon binds to components of inducer molecules. It is therefore quite possible that direct interaction of interferon with some RNA sequences could play a role in interferon induction. "Spontaneous" interferon production has been observed in many cells, and interferon may be present in very small amounts in all cells as a constitutive protein. The appearance in the cell of polynucleotides that bind interferon could prevent the latter from exerting its normal function, and hence stimulate the cell to make more interferon. This hypothesis could explain why so many RNAs can act as interferon "inducers" with various efficiencies. All these RNAs may have more or less specific sequences with affinity for interferon and therefore be more or less efficient in triggering its synthesis.

The interaction of interferon molecules with RNA polymers

also offers an interesting avenue for the purification of interferon by affinity chromatography on these polymers because, in a single run on a poly(U)-Sephadex column, crude interferon was purified to a specific activity of  $2 \times 10^9$  units/mg of protein.

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