Interferon Induction by Poly(inosinic acid)·Poly(cytidylic acid) Segmented by Spin-Labels†

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ABSTRACT: Poly(inosinic acid)·poly(cytidylic acid) [$(I)_n$·$(C)_n$] duplexes of which the $(C)_n$ strand was modified to various degrees chemically or enzymatically with nitroxide radicals (spin-labels) were evaluated for interferon-inducing activity. Upon annealing of the chemically modified $(C)_n$, $(lC,C_x)_n$ (x = 1000 or 16), with $(I)_n$, the interferon-inducing activity was similar to that of $(I)_n$·$(C)_n$ in PRK cell cultures. However, to overcome hydrolysis of the spin-label linkage in $(lC,C_x)_n$, an enzymatic approach was taken to synthesize $(ls^4U,C_x)_n$ copolymers with x = 100, 38, 16, and 8. The $(ls^4U,C_x)_n$ copolymers were chemically stable, and upon annealing with $(I)_n$ the correlation time of the nitroxide moiety in $(I)_n$·$(ls^4U,C_x)_n$ was determined. A comparison of this correlation time with that measured for $(RUGT,U_{100})_n$·$(A)_n$, which contains the nitroxide moiety in position 5 of the U moiety, suggests that the ls^4U residue is in a nonintrahelical conformation and partitions the duplex into double-helical segments of varying size. The interferon-inducing activity of $(I)_n$·$(ls^4U,C_x)_n$ was evaluated in primary rabbit kidney, human skin fibroblast (strain VGS), and mouse L-929 cell cultures as well as in rabbits. The ls^4U residue did not cause a significant change in the interferon induction as compared to $(I)_n$·$(C)_n$ in most systems tested unless $x < 16$. These findings indicate that double-helical segments of ~16 base pairs partitioned by nonintrahelical ls^4U residues suffice to trigger the interferon response in all systems studied.

Circular dichroism studies of base-modified polynucleotide duplexes have shown that alterations in the helicity of the duplex are accompanied by changes in interferon-inducing activity (Bobst et al., 1976). The concept that the induction of interferon by duplexes strongly depends on the recognition of their topology (De Clercq et al., 1974; Torrence et al., 1975; Torrence & De Clercq, 1977) was reinforced by results showing that the $(dIn_3)_n$·$(C)_n$[1] (De Clercq et al., 1978), $(dIc1)_n$·$(C)_n$, and $(dIf1)_n$·$(C)_n$ (De Clercq et al., 1980) analogues of $(I)_n$·$(C)_n$ are active interferon inducers. Apparently, the substitution of the 2′-OH by a 2′-azido, a 2′-chloro, or a 2′-fluoro in the $(I)_n$ strand of $(I)_n$·$(C)_n$ results in a topology which is also recognized by the interferon-inducer receptor, and it is not surprising that the circular dichroism spectra of $(dIn_3)_n$·$(C)_n$ and of $(I)_n$·$(C)_n$ were shown to be similar (Fukui et al., 1977). Evidence has also been presented (De Clercq et al., 1974, 1975; Greene et al., 1978) to suggest that nucleic acid binding to the interferon-inducer receptor is not the only critical event for interferon induction. Various polynucleotides, inactive as inducers, may compete with active inducers. In addition, 2′-*O*-methyl modification of $(I)_n$·$(C)_n$ gave duplexes with similar topology to $(I)_n$·$(C)_n$ which could compete with $(I)_n$·$(C)_n$ for the putative receptor but were inactive as inducers. From interferon-induction experiments with 2′-*O*-methyl modified $(I)_n$·$(C)_n$ (Greene et al., 1978) and with mismatched $(I_x,U)_n$·$(C)_n$ analogues (De Clercq et al., 1979), the suggestion has been advanced that interferon induction is the result of a biphasic event consisting of an initial binding of a high molecular weight duplex with proper topology followed by an induction sequence dependent on 6–12 base pairs.

In view of the demonstrated usefulness of spin-labeled nucleic acids to monitor nucleic acid–tissue cell surface interactions (Kouidou et al., 1978), it was found desirable to investigate the effects of nitroxide radicals incorporated in nucleic acid duplexes on interferon-inducing activity. For that reason we evaluated the interferon-inducing activity of $(I)_n$·$(C)_n$ duplexes of which the $(C)_n$ strand had been modified chemically or enzymatically with nitroxide radicals. The $(lC,C_{1000})_n$ and $(lC,C_{16})_n$ strands were made chemically, whereas the $(ls^4U,C_x)_n$ strands were obtained enzymatically. The interferon-inducing activity was found to remain essentially unchanged in the various systems tested with $(I)_n$·$(C)_n$ duplexes containing no less than ~16 uninterrupted base pairs. The data on the mismatched $(I)_n$·$(ls^4U,C_x)_n$ duplexes suggest the involvement of about one helical turn to trigger the interferon response as part of a postulated biphasic concept of interferon induction.

Materials and Methods

All nucleic acids were purified through Sephacryl S-200 (1.5 × 78 cm). The molecular weight and homogeneity were assessed by disc gel electrophoresis using the $(C)_n$ molecular size kit of P-L Biochemicals as standards. All polynucleotide samples used for this study had a weight-average molecular weight of 100 000–200 000. $(I)_n$ and $(C)_n$ were bought from P-L Biochemicals; pancreatic ribonuclease A (Type 1-A), s^4UDP, CDP, and UDP were obtained from Sigma Chemical Co. ls^4UDP was synthesized by reacting 4-(α-iodoacetamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy and 4-thiouridine 5′-diphosphate and purifying the reaction mixture according

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[1] Abbreviations used: $(I)_n$, poly(inosinic acid); $(C)_n$, poly(cytidylic acid); in $(I)_n$·$(C)_n$ P-L, P-L stands for Pabst Laboratories; $(lC,C_x)_n$, poly(cytidylic acid) chemically modified with the anhydride of 3-carboxy-2,2,5,5-tetramethylpyrrolinyl-1-oxy; $(ls^4U,C_x)_n$, a spin-labeled nucleic acid obtained through enzymatic copolymerization of ls^4UDP and cytidine 5′-diphosphate; ls^4UDP, *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)-*S*-[1-(5′-*O*-diphosphono-β-D-ribofuranosyl)uracil-4-yl]thioglycolamide; RUGT, *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)-*O*-(1-β-D-ribofuranosyluracil-5-yl)glycolamide; T_m^{OD}, melting temperature determined by UV spectroscopy; T_m^{sp}, melting temperature determined by ESR spectroscopy by plotting −log τ against $1/T$; T_1^{sp}, the initial temperature of the rapid change in correlation time in the plot of −log τ vs. $1/T$; τ, correlation time; PRK, primary rabbit kidney; HSF (VGS), human skin fibroblast (strain VGS); VSV, vesicular stomatitis virus; DEAE, diethylaminoethyl; RNA, ribonucleic acid.

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to a published procedure used for UDP spin-labeled in position 5 (Hakam et al., 1979). $(lC,C_x)_n$ was obtained by chemical modification of $(C)_n$ with the anhydride of 3-carboxy-2,2,5,5-tetramethylpyrrolinyl-1-oxy, and $(ls^4U,C_x)_n$ was synthesized enzymatically by using *Micrcoccus luteus* polynucleotide phosphorylase at various input ratios of ls^4UDP and CDP. As observed by ESR, the distribution of the spin-labeled nucleotides in $(ls^4U,C_x)_n$ is random since no broadening and signal intensity loss due to Heisenberg spin exchange is observed with $x \geq 16$. Spin exchange is only noted with $x = 8$ and is attributed to the proximity of the spin-label residues. A detailed description of the chemistry of $(lC,C_x)_n$ and $(ls^4U,C_x)_n$ will be published elsewhere.

The UV absorbance thermal denaturation studies were done with a Gilford 250 spectrophotometer and a Gilford 2527 thermoprogrammer with a cell assembly consisting of four quartz microcells in an electrically heated block at a heating rate of 1 °C/min. The ESR thermal melting studies were done with a Varian E-4 spectrometer interfaced to a Fabri-Tek 1074 instrument computer in a flat quartz microcell. A detailed description for such measurements was given elsewhere (Bobst, 1979).

The methodology for measuring interferon production in PRK, HSF (VGS), L-929 cells, and intact rabbits has been described previously (De Clercq et al., 1976; De Clercq, 1981).

PRK Cells. When confluent, PRK cells were exposed for 1 h to the polynucleotide in serum-free Eagle's minimal essential medium (MEM) and subsequently treated with cycloheximide (2 μg/mL) for 3 h and actinomycin D (3 μg/mL) for 30 min. Following this "superinduction" period, the cells were washed extensively with MEM and further incubated for 18–20 h with MEM containing 3% heated fetal calf serum (FCS). The cell culture fluid was then harvested and assayed for interferon by the viral cytopathogenicity (CPE)–inhibition technique in PRK cells using vesicular stomatitis virus (VSV) as the challenge virus.

HSF Cells. For HSF cells (strain VGS; Billiau et al., 1973) a similar superinduction procedure was employed, except that cycloheximide (10 μg/mL) was left in contact with the cells for 6 h and actinomycin D (1 μg/mL) was present during the last 2 h of this 6-h incubation period. Interferon titrations were carried out in HSF (VGS) cells by using a VSV CPE–inhibition technique similar to that used for the rabbit interferon titrations.

L-929 Cells (Procedure A). Mouse L-929 cell cultures were pretreated ("primed") for 20 h with mouse interferon [100 units/mL of MEM (+3% FCS)], exposed to the polynucleotide (in serum-free MEM) for 1 h, and further incubated for 20–24 h with MEM + 3% FCS. The cell culture fluid was then harvested and assayed for interferon by a VSV plaque-reduction technique in L-929 cells.

L-929 Cells (Procedure B). Mouse L-929 cell cultures were pretreated for 1 h with DEAE-dextran [250 μg/mL of MEM (serum-free)], exposed to the polynucleotide (in serum-free MEM) for 1 h, and further processed as described in procedure A.

Rabbits. Young rabbits weighing ~1 kg were injected intravenously with the indicated amount of polynucleotide (dissolved in 1 mL of phosphate-buffered NaCl). Blood samples were taken at 1, 2, 4, and 8 h after injection, and the serum was assayed for interferon by measuring the inhibition of VSV CPE in PRK cells.

Results

The data for interferon induction with chemically spin-labeled $(I)_n·(lC,C_x)_n$ in PRK cell cultures are shown in Table I. Interferon induction by $(I)_n·(lC,C_x)_n$ appears to be similar to that of unmodified $(I)_n·(C)_n$. However, when the stability of the linkage between the nitroxide moiety and the $(C)_n$ strand in $(lC,C_x)_n$ was examined with a poly(L-lysine) solution of known concentration, some release of the nitroxide radicals from the $(lC,C_x)_n$ was revealed. This poly(L-lysine) test, which is often referred to as the free-spin test (Bobst, 1979), suggests that acyl linkage between the nitroxide moiety and the C residue is readily hydrolyzed.

Table I: Interferon Induction in PRK Cell Cultures Superinduced with Cycloheximide and Actinomycin D by Chemically Spin-Labeled $(I)_n·(lC,C_x)_n$

double-stranded system	interferon titer (log units/mL) at polymer concn (μmol/mL) 0.0025	0.025	0.25
$(I)_n·(C)_n$	2.8	3.5	4.0
$(I)_n·(lC,C_{1000})_n$	2.8	3.5	4.3
$(I)_n·(lC,C_{16})_n$	3.5	3.5	4.0
$(I)_n·(C)_n$, P-L Biochemical	2.8	3.5	3.8

A different route was designed to prepare $(C)_n$ analogues with a more stable covalent linkage between the nitroxide moiety and the nucleotide, in order to overcome the hydrolysis problem. This was accomplished by synthesizing the spin-labeled nucleoside diphosphate ppls^{4}U and subsequently incorporating it enzymatically to yield $(ls^4U,C_x)_n$ copolymers. The free-spin test revealed that the $(ls^4U,C_x)_n$ strands were stable, i.e., free nitroxide radicals were not released over a period of days at pH 7. In view of the chemical stability of the various $(ls^4U,C_x)_n$ synthesized enzymatically, the physicochemical characteristics of $(ls^4U,C_{100})_n$, $(ls^4U,C_{38})_n$, $(ls^4U,C_{16})_n$, and $(ls^4U,C_8)_n$ were determined in the single-stranded form as well as after complex formation with $(I)_n$. In addition, with the elimination of the risk of release of the nitroxide radical, the various $(I)_n·(ls^4U,C_x)_n$ duplexes were tested as potential interferon inducers in the following five assay systems: PRK cells superinduced with cycloheximide and actinomycin D; HSF cells (VGS) superinduced with cycloheximide and actinomycin D; mouse L-929 cells primed with mouse interferon; mouse L-929 cells pretreated with DEAE-dextran; intact rabbits.

In Figure 1A the optical density melting of $(I)_n·(ls^4U,C_{38})_n$ is compared with that of $(I)_n·(C)_n$. It is apparent that the T_m^{OD} values and the hypochromicities are very similar for both compounds indicating that the perturbation by the spin-label is insufficient to detect a loss of hydrogen bonding and base stacking by absorbance measurements. The spin melting of $(ls^4U,C_{38})_n$ and $(I)_n·(ls^4U,C_{38})_n$ is shown in the form of Arrhenius plots in Figure 1B. Correlation times of the nitroxide radicals were calculated with the formalism of Kivelson (1960). Discontinuities as observed and discussed for other spin-labeled duplexes are noticeable (Pan & Bobst, 1973; Bobst, 1980). The T_1^{sp} and T_m^{sp} are 52 and 59 °C, respectively (Table II), and indicate that the residues contiguous to the ls^4U remain base paired at the temperature of the interferon-induction assays.

In Figure 1C the optical density melting profile of the duplex $(I)_n·(ls^4U,C_{16})_n$ is shown together with that of $(I)_n·(C)_n$. The ls^4U residue causes some noticeable perturbation with respect to cooperativity of the transition, hypochromicity, and T_m^{OD} value. The hypochromicity is reduced by several percent, and the T_m^{OD} is lowered by ~6 °C. Figure 1D shows the spin melting profile of $(ls^4U,C_{16})_n$ and of $(I)_n·(ls^4U,C_{16})_n$, which reveals segment patterns similar to those seen in Figure 1B. Of interest here is the T_1^{sp} value of 42 °C (Table II) which

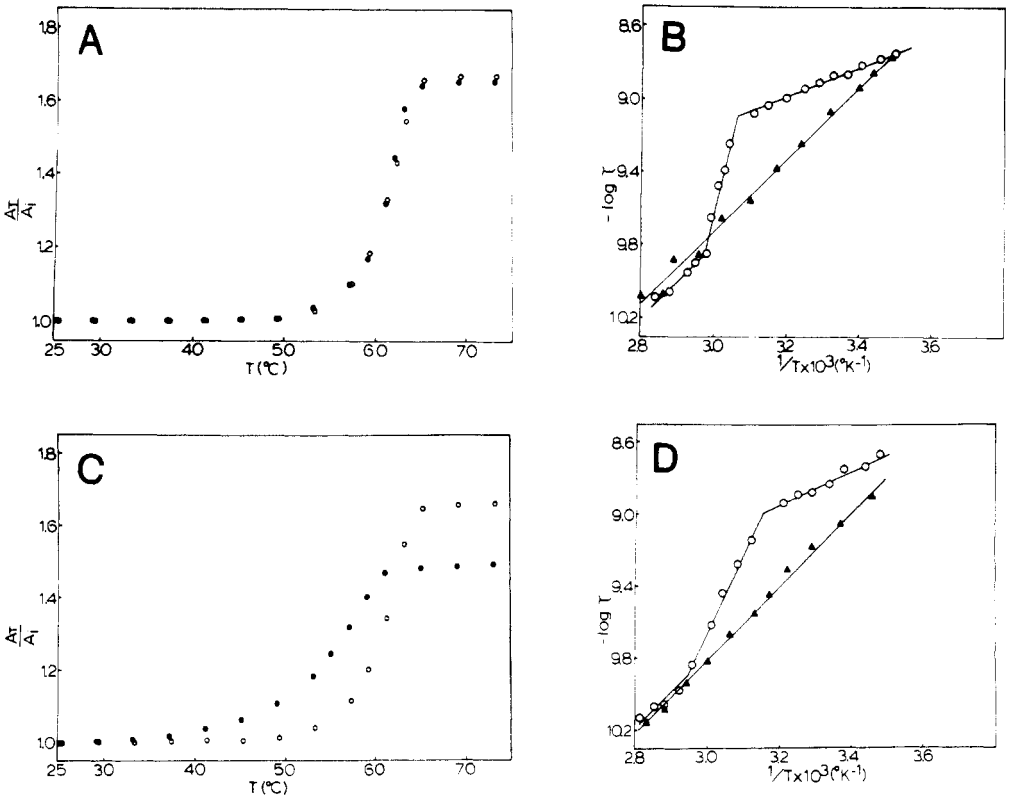

FIGURE 1: (A) Normalized UV absorbance profiles of $(I)_n \cdot (C)_n$ (○) and $(I)_n \cdot (ls^4U,C_{38})_n$ (●) in 0.14 M NaCl and 0.01 M sodium cacodylate buffer, pH 7, at a nucleotide residue concentration of 2.8×10^{-4} M. (B) Dependence of $-\log \tau$ on the inverse absolute temperature for $(ls^4U,C_{38})_n$ (▲) and $(I)_n \cdot (ls^4U,C_{38})_n$ (○) in 0.14 M NaCl and 0.01 M sodium cacodylate buffer, pH 7. The nucleotide residue concentration was in both cases 2.8×10^{-4} M. (C) Normalized UV absorbance profiles of $(I)_n \cdot (C)_n$ (○) and $(I)_n \cdot (ls^4U,C_{16})_n$ (●) in 0.14 M NaCl and 0.01 M sodium cacodylate buffer, pH 7, at a nucleotide residue concentration of 1.45×10^{-4} M. (D) Dependence of $-\log \tau$ on the inverse absolute temperature for $(ls^4U,C_{16})_n$ (▲) and $(I)_n \cdot (ls^4U,C_{16})_n$ (○) in 0.14 M NaCl and 0.01 M sodium cacodylate buffer, pH 7. The nucleotide residue concentration was in both cases 2.8×10^{-4} M.

Table II: Effect of Percent Spin-Labeling on T_m^{OD} [a] and T_m^{sp} [a] of $(I)_n \cdot (ls^4U,C_x)_n$

double-stranded system	T_m^{OD} (±0.5 °C)	% hypochromicity (±2)	T_1^{sp} [c] (±0.5 °C)	T_m^{sp} [d] (±0.5 °C)
$(I)_n \cdot (C)_n$	61	40	–	–
$(I)_n \cdot (ls^4U,C_{100})_n$	61	40	*b*	*b*
$(I)_n \cdot (ls^4U,C_{38})_n$	61	40	52	59
$(I)_n \cdot (ls^4U,C_{16})_n$	55	33	42	55
$(I)_n \cdot (ls^4U,C_8)_n$	50	28	*b*	*b*

[a] In 0.14 M NaCl and 0.01 M sodium cacodylate, pH 7. [b] Not determined. [c] T_1^{sp} is the temperature at which the correlation time starts to increase rapidly. [d] T_m^{sp} is the temperature at the midpoint of the discontinuity of the low- and high-temperature segments.

Table III: Induction of Cellular Resistance to Virus Infection in HSF (VGS) Cell Cultures by Spin-Labeled $(I)_n \cdot (C)_n$

polynucleotide	minimum inhibitory concn[a] (nmol/mL)
$(I)_n \cdot (C)_n$	0.4
$(I)_n \cdot (ls^4U,C_{100})_n$	0.4
$(I)_n \cdot (ls^4U,C_{38})_n$	0.8
$(I)_n \cdot (ls^4U,C_{16})_n$	1
$(I)_n \cdot (ls^4U,C_8)_n$	>25
$(I)_n \cdot (C)_n$ P-L	0.1

[a] Required to reduce VSV-induced cytopathogenicity by 50%. The polynucleotides were added in serum-free medium 24 h prior to VSV challenge. Average values for two experiments.

is higher than the temperature at which the interferon induction assays are carried out. From Table II it is also apparent that the T_m^{OD} and the percent hypochromicity are considerably lower for $(I)_n \cdot (ls^4U,C_{16})_n$ than for the unmodified duplex.

The interferon-inducing activity of $(I)_n \cdot (ls^4U,C_x)_n$ was tested in various biological systems. The activity of the single strands was not evaluated, but it is postulated that $(ls^4U,C_x)_n$ or $(I)_n$ alone would not be active at the concentrations used for the double-stranded complexes. The activity of $(ls^4U,C_x)_n$ in PRK cell cultures superinduced with cycloheximide and actinomycin D is shown with Figure 2A. It is apparent that in this system the interferon-inducing activity is comparable for all spin-labeled $(I)_n \cdot (C)_n$ derivatives tested and essentially the same for the derivatives as for the unlabeled duplex. In the assay systems of HSF cells (VGS) superinduced with cycloheximide and actinomycin D (Figure 2B) and of mouse L-929 cells primed with mouse interferon (Figure 2C), the interferon inducers $(I)_n \cdot (ls^4U,C_x)_n$ with $x \geq 16$ displayed again essentially the same activity as $(I)_n \cdot (C)_n$, whereas the activity of $(I)_n \cdot (ls^4U,C_8)_n$ fell below the level of detection.

The perturbation effect of the ls^4U building block became more apparent in mouse L-929 cells pretreated with DEAE-dextran (Figure 2D) and in rabbits (Figure 3), since in these systems $(I)_n \cdot (ls^4U,C_{16})_n$ was less active as an interferon inducer than $(I)_n \cdot (ls^4U,C_{38})_n$, $(I)_n \cdot (ls^4U,C_{100})_n$, and $(I)_n \cdot (C)_n$.

The susceptibility of the various spin-labeled duplexes to degradation by pancreatic ribonuclease A was monitored by residual interferon-inducing activity in PRK cells as a function of the amount of ls^4U incorporated into the $(C)_n$ strand. The data are shown in Figure 4, and it seems that the resistance to degradation decreases in the following order: $(I)_n \cdot (C)_n \simeq (I)_n \cdot (ls^4U,C_{100})_n \simeq (I)_n \cdot (ls^4U,C_{38})_n > (I)_n \cdot (ls^4U,C_{16})_n > (I)_n \cdot (ls^4U,C_8)_n$.

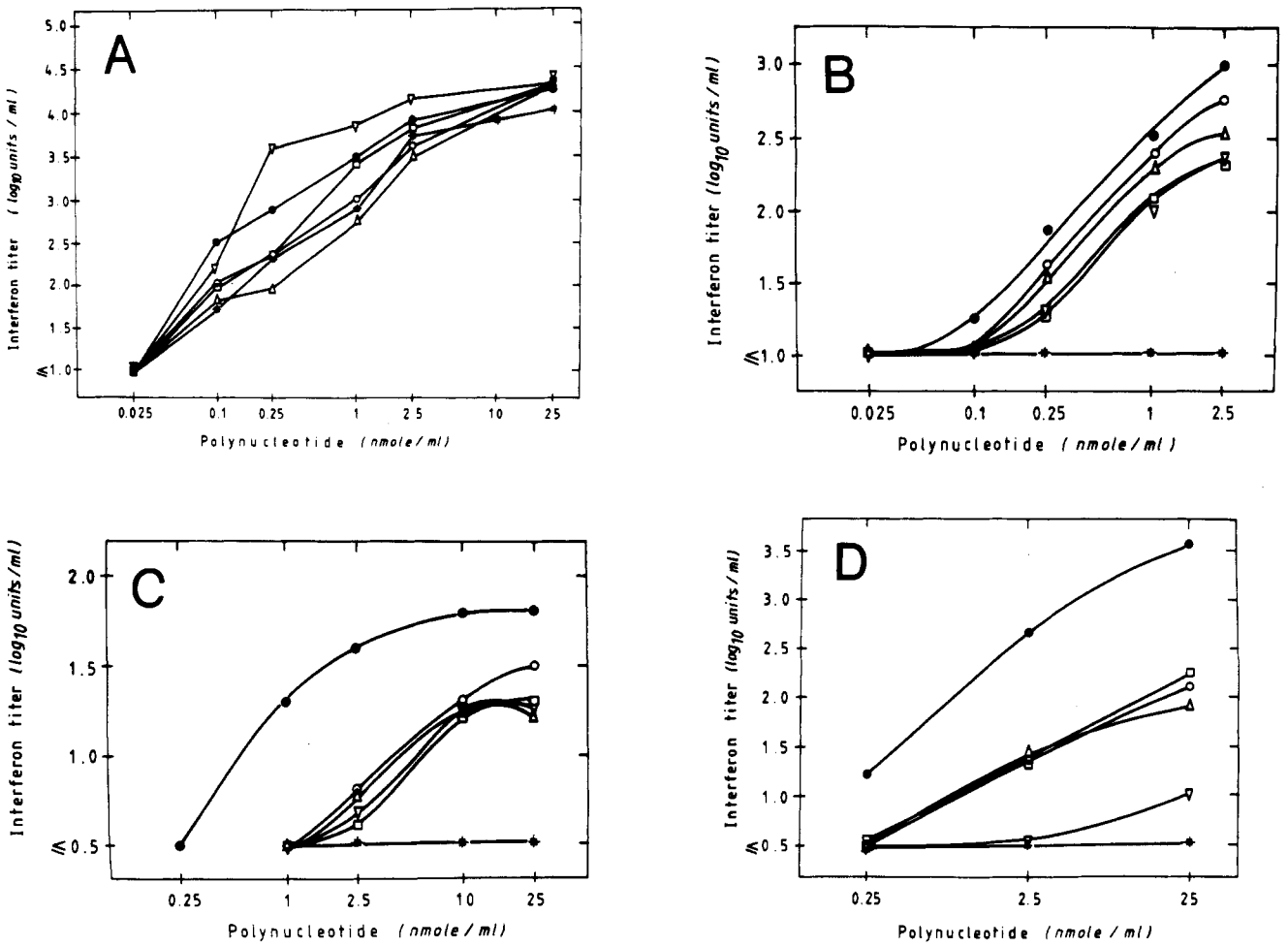

FIGURE 2: (A) Interferon-inducing activity of $(I)_n \cdot (C)_n$ (○), $(I)_n \cdot (ls^4U,C_{100})_n$ (△), $(I)_n \cdot (ls^4U,C_{38})_n$ (□), $(I)_n \cdot (ls^4U,C_{16})_n$ (▽), and $(I)_n \cdot (ls^4U,C_8)_n$ (*) in primary rabbit kidney (PRK) cell cultures superinduced with cycloheximide and actinomycin D. (●) $(I)_n \cdot (C)_n$ P-L. Average values for six experiments. (B) Interferon-inducing activity of $(I)_n \cdot (C)_n$ (○), $(I)_n \cdot (ls^4U,C_{100})_n$ (△), $(I)_n \cdot (ls^4U,C_{38})_n$ (□), $(I)_n \cdot (ls^4U,C_{16})_n$ (▽), and $(I)_n \cdot (ls^4U,C_8)_n$ (*) in human skin fibroblast (HSF) cell cultures (VGS) superinduced with cycloheximide and actinomycin D. (●) $(I)_n \cdot (C)_n$ P-L. Average values for three experiments. (C) Interferon-inducing activity of $(I)_n \cdot (C)_n$ (○), $(I)_n \cdot (ls^4U,C_{100})_n$ (△), $(I)_n \cdot (ls^4U,C_{38})_n$ (□), $(I)_n \cdot (ls^4U,C_{16})_n$ (▽), and $(I)_n \cdot (ls^4U,C_8)_n$ (*) in mouse L-929 cell cultures primed with mouse interferon (100 units/mL). (●) $(I)_n \cdot (C)_n$ P-L. Average values for two experiments. (D) Interferon-inducing activity of $(I)_n \cdot (C)_n$ (○), $(I)_n \cdot (ls^4U,C_{100})_n$ (△), $(I)_n \cdot (ls^4U,C_{38})_n$ (□), $(I)_n \cdot (ls^4U,C_{16})_n$ (▽), and $(I)_n \cdot (ls^4U,C_8)_n$ (*) in mouse L-929 cell cultures pretreated with DEAE-dextran (250 μg/mL). (●) $(I)_n \cdot (C)_n$ P-L.

Finally, in Table III the minimum inhibitory concentrations required to reduce VSV-induced cytopathogenicity by 50% are given for the enzymatically spin-labeled duplexes $(I)_n \cdot (ls^4U,C_x)_n$. There was no evidence of polynucleotide cytotoxicity, even at the highest polynucleotide concentration tested (25 nmol/mL), as based on microscopic appearance of the cell morphology and the development of viral cytopathogenicity in these cells. The data clearly indicate that the biological activity remains essentially the same with $x \geq 16$, whereas with $x = 8$ a considerably higher polymer concentration is required to inhibit viral cytopathogenicity.

Discussion

The observation that interferon-inducing activity with chemically spin-labeled $(I)_n \cdot (lC,C_x)_n$ was similar for various values of x provided the impetus for pursuing the spin-labeling approach further. However, because of the ease with which the nitroxide radicals were released from the $(I)_n \cdot (lC,C_x)_n$ duplexes, no attempts were made to characterize the physicochemical characteristics of these duplexes. In contrast, the enzymatically spin-labeled inducers were thoroughly analyzed by UV and ESR spectroscopy since the nitroxide moiety remains attached to the duplexes. Incorporation of a noncomplementary base such as ls^4U in the $(C)_n$ strand could result in $(I)_n \cdot (ls^4U,C_x)_n$ helices in which the mismatched bases are accommodated intra- or extrahelically. Opposing noncomplementary pyrimidine–purine pairs can assume intrahelical conformations, if the helical environment (temperature, ionic strength, etc.) and the quantity of noncomplementary bases are properly chosen (Wang & Kallenbach, 1971). The ambiguity as to whether the ls^4U base is extra- or intrahelical does not seem to exist for $(I)_n \cdot (ls^4U,C_x)_n$ in view of ESR results. Namely, τ values obtained by Kivelson's formalism are similar for all $(I)_n \cdot (ls^4U,C_x)_n$ studied at 37 °C ($\tau = (1.2 \pm 0.1) \times 10^{-9}$ s), but these values are significantly lower than the one calculated for the duplex $(A)_n \cdot (RUGT,U_{100})_n$ ($\tau = (1.6 \pm 0.1) \times 10^{-9}$ s) (Langemeier & Bobst, 1981). In particular, it should be emphasized that the correlation times, τ, of $(RUGT,U_{100})_n$ and $(ls^4U,C_x)_n$ at 37 °C are $(2.4 \pm 0.2) \times 10^{-10}$ and $(4.4 \pm 0.2) \times 10^{-10}$ s, respectively. These differences are readily explained by taking into account the differences in base stacking of the two single strands. Upon annealing of the labeled single strands, however, the motion of the label should be dominated by the topology of the duplex. The considerably smaller decrease in mobility of the nitroxide moiety (0.8×10^{-9} s) upon annealing of $(I)_n$ with $(ls^4U,C_x)_n$ as compared to the $(A)_n \cdot (RUGT,U_{100})_n$ system with a difference of 1.4×10^{-9} s strongly suggests that the ls^4U moiety is present in a nonintrahelical conformation. Such a conformation allows more freedom of motion for the spin-label than an intrahelical one

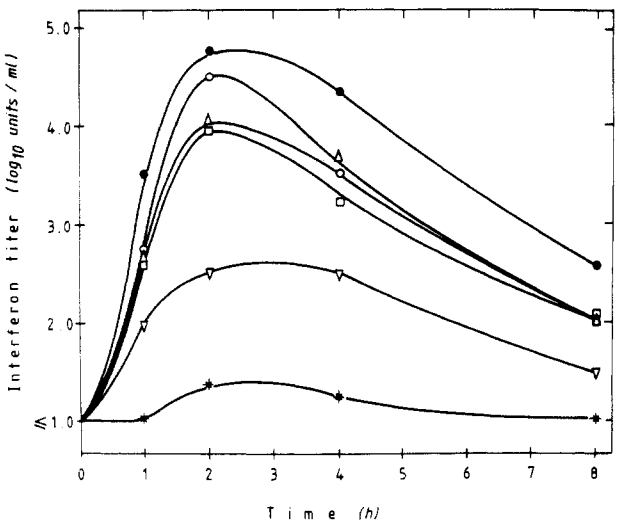

FIGURE 3: Interferon-inducing activity of $(I)_n \cdot (C)_n$ (○), $(I)_n \cdot (ls^4U,C_{100})_n$ (△), $(I)_n \cdot (ls^4U,C_{38})_n$ (□), $(I)_n \cdot (ls^4U,C_{16})_n$ (▽), and $(I)_n \cdot (ls^4U,C_8)_n$ (*) in rabbits. Serum interferon titers were measured at 1, 2, 4, and 8 h after intravenous injection of 10 nmol of polynucleotide/mL per rabbit. (●) $(I)_n \cdot (C)_n$ P-L.

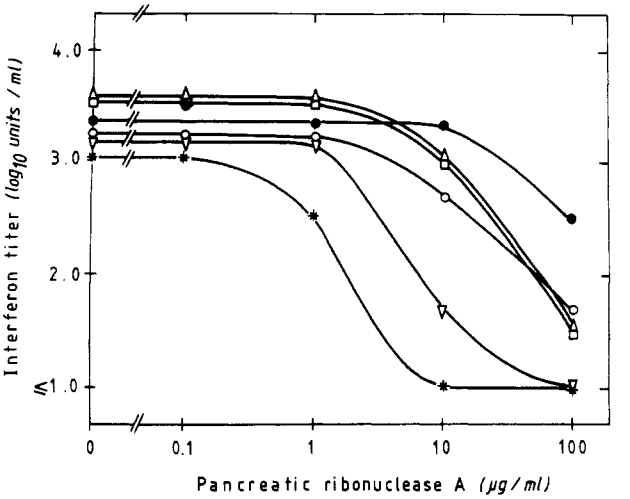

FIGURE 4: Susceptibility of $(I)_n \cdot (C)_n$ (○), $(I)_n \cdot (ls^4U,C_{100})_n$ (△), $(I)_n \cdot (ls^4U,C_{38})_n$ (□), $(I)_n \cdot (ls^4U,C_{16})_n$ (▽), and $(I)_n \cdot (ls^4U,C_8)_n$ (*) to degradation by pancreatic ribonuclease A, as monitored by the interferon-inducing activity of the polynucleotide–enzyme mixtures in PRK cell cultures superinduced with cycloheximide and actinomycin D. The polynucleotides were incubated at 1 nmol/mL with various concentrations of pancreatic ribonuclease (as indicated) for 1 h at 37 °C and then assayed for interferon-inducing activity. (●) $(I)_n \cdot (C)_n$ P-L.

which would put the nitroxide moiety essentially into the major groove of the duplex. Thus, the nonintrahelical ls^4U moiety in $(I)_n \cdot (ls^4U,C_x)_n$ produces small double-helical segments of varying size.

The similarity in interferon-inducing activity of $(I)_n \cdot (ls^4U,C_{100})_n$, $(I)_n \cdot (ls^4U,C_{38})_n$, and $(I)_n \cdot (C)_n$ is most probably due to the length of the partitioned double-helical segments. It is interesting to note that the interferon-inducing activity of the $(I)_n \cdot (ls^4U,C_x)_n$ with $x \leq 16$ varied considerably from one assay system to another. These differences are particularly evident if one compares the data of Figure 2A with those shown in Figure 2D. In PRK cells strongly and weakly spin-labeled $(I)_n \cdot (C)_n$ derivatives exhibited similar interferon-inducing activity, whereas $(I)_n \cdot (ls^4U,C_x)_n$ with $x \leq 16$ showed little activity in mouse L-929 cells pretreated with DEAE-dextran. Thus, structural requirements of interferon inducers that are deduced from one induction system may not necessarily hold for the other induction system, as has already been pointed out earlier (De Clercq et al., 1978).

The results presented in Figure 4 may be interpreted as the consequence of the action of pancreatic ribonuclease A during the 1-h incubation period (in the absence of the cells), since polynucleotides are known to attach very rapidly to the cells and to trigger the interferon-induction process within a few minutes after exposure to the cells. Thus, the data of residual interferon-inducing activity in PRK cells suggest that the susceptibility of $(I)_n \cdot (ls^4U,C_x)_n$ to pancreatic ribonuclease A digestion increases with a decreasing value of x. This is supported by a UV hydrolysis study done on a series of mismatched duplexes of $(I,U)_n$ and $(C,U)_n$ copolymers with $(C)_n$ and $(I)_n$, respectively, which had been exposed to pancreatic ribonuclease (Carter et al., 1972). Our observed parallelism points to a close, if not casual, relationship between the interferon-inducing potency of the duplexes and their resistance to ribonuclease A. Ribonuclease A has been shown to selectively degrade the $(C)_n$ strand of $(I)_n \cdot (C)_n$ (De Clercq, 1979).

In conclusion, the relative order of decreasing interferon-inducing activity was $(I)_n \cdot (C)_n \geq (I)_n \cdot (ls^4U,C_{100})_n \geq (I)_n \cdot (ls^4U,C_{38})_n > (I)_n \cdot ls^4U,C_{16})_n > (I)_n \cdot (ls^4U,C_8)_n$. One may contend that the ls^4U moieties did not cause a dramatic change in the interferon-inducing activity of $(I)_n \cdot (ls^4U,C_x)_n$, unless x went from 16 to 8, as most typically shown with Figure 2B,C. These findings suggest that double-helical segments of ~16 base pairs partitioned by nonintrahelical residues suffice to trigger the interferon response in various systems and also support some earlier hypotheses (Greene et al., 1978; De Clercq et al., 1979) that about one double helical turn of an RNA duplex is required for interferon induction.

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Spectroscopic Studies on Acetylcholinesterase: Influence of Peripheral-Site Occupation on Active-Center Conformation†

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ABSTRACT: Each subunit of acetylcholinesterase (AchE) contains, in addition to an active center, a peripheral anionic site at which a variety of structurally diverse cationic ligands associate. The influence of peripheral-site occupation on active-center conformation was assessed by examination of (pyrenebutyl methylphosphono)acetylcholinesterase (PBMP–AchE). In this conjugate, the pyrenebutyl moiety is linked covalently through a phosphonyl unit to the active-center serine. Association of propidium at the peripheral site results in quenching of ~88% of the pyrenebutyl fluorescence through dipolar excitation transfer; subsequent dissociation of propidium by competitive peripheral site ligands (e.g., gallamine, decamethonium, and *d*-tubocurarine) results in restoration of the pyrenebutyl fluorescence. Direct monitoring of propidium fluorescence provides an alternative demonstration of peripheral-site occupation and estimation of ligand affinity. The values of the dissociation constants calculated for gallamine and *d*-tubocurarine with PBMP–AchE are virtually equivalent with those determined for the native enzyme. In contrast, the association of decamethonium with PBMP–AchE is found to occur with a 50-fold lower affinity than with the native AchE; the decrease in affinity likely reflects steric occlusion of the active center by the bulky pyrenebutyl group. For the association of *d*-tubocurarine, although the competitive titration profile determined from the measurement of the propidium fluorescence is consistent with binding at a single high-affinity site, the titration profile determined through measurement of the pyrenebutyl fluorescence reveals an additional site of *d*-tubocurarine association. In the presence of low concentrations of *d*-tubocurarine, the fluorescence quantum yield of pyrenebutyl fluorescence is found to increase, whereas at higher ligand concentrations, pyrenebutyl fluorescence is quenched. Occupation of the high-affinity site which is competitive with propidium is required prior to occupation of the lower affinity site. The pyrenebutyl absorption spectrum of PBMP–AchE is shifted to longer wavelengths in the presence of gallamine and low concentrations of *d*-tubocurarine. Such spectral changes appear to be associated with binding exclusively at the peripheral anionic site. In the presence of decamethonium and high concentrations of *d*-tubocurarine, the pyrenebutyl spectrum undergoes striking alterations, and since under these conditions these ligands bind at the active center, the spectral perturbations likely reflect association of the cationic moieties within the vicinity of the active center. The intense optical activity exhibited in the circular dichroism spectra of the pyrenebutyl moiety of PBMP–AchE ($[\theta]_{348} = -4 \times 10^4$ deg cm^2/dmol) suggests that the chromophore is in apposition with a highly dissymmetric protein surface and that their relative orientations are dramatically altered upon occupation of either the peripheral site or the active center. These independent spectroscopic approaches reveal changes in active-site conformation that are associated with occupation of the spatially distinct peripheral anionic site and provide spectroscopic indexes which allow one to distinguish the site of ligand occupation.

Acetylcholinesterase (AchE)[1] exhibits a remarkable capacity to bind structurally diverse cationic ligands. In addition to the class of ligands which bind at the active center, there exists another class which associates at an anionic locus remote from the active center (Changeux, 1966). Although these ligands, which include the structures of gallamine, *d*-tubocurarine, and decamethonium, bind in a mutually exclusive manner at the peripheral anionic site, their modes of inhibition of catalysis are not equivalent and appear to be a property of the ligand as well as the site of occupation. Such behavior may suggest that either the occupation of the peripheral site by different ligands induces different active-center conformations or the peripheral site comprises a matrix of nonequivalent loci, all remote from the enzyme active center.

Spectroscopic probes have been employed to examine the microscopic environment and conformation of proteins (Stryer, 1968). When covalently situated at a distinct site, these probes

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[1] Abbreviations used: AchE, acetylcholinesterase; PBMPF, pyrenebutyl methylphosphonofluoridate; CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; dansyl, 8-(dimethylamino)-1-naphthalenesulfonate.

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