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STRUCTURES OF POLY(dA-dT,ip^{5}dU) CONTAINING VARIOUS SMALL AMOUNTS OF THE ANTIHERPETIC 5-ISOPROPYL-2'-DEOXYURIDINE

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Three different concentrations of the antiherpetic agent 5-isopropyl-2'-deoxyuridine (ip^{5}dU) were introduced into the synthetic DNA poly(dA-dT) to analyze resulting copolymers by electron microscopy, UV absorption and CD spectroscopy. The poly(dA-dT,ip^{5}dU) containing 1.3 and 4.3% ip^{5}dU did not much differ from the parent poly(dA-dT) but poly(dA-dT,ip^{5}dU) with 7.1% ip^{5}dU behaved in an unusual way. Results are explained by the notion that if bulky isopropyls occur sufficiently close to each other then stable hairpins protruding from the double helix are formed, presumably to accomodate the ip^{5}dU-s into the loops.

The thymidine analogue 5-*iso*propyl-2'-deoxyuridine (ip^{5}dU) is a commercially available drug (HEVIZOS) (1) for the local treatment of patients with herpes labialis, zoster or progenitalis (2). Antiherpes action of 5-substituted dU-s at the molecular level is assumed to originate from i/ the inhibition of viral DNA polymerase by the modified triphosphate formed in the virus-infected cells by the herpes-encoded thymidine kinase, and ii/ from the altered biochemical properties of the newly synthesized herpesvirus DNA containing incorporated 5-substituted dUMP

ABBREVIATIONS
5-isopropyl-2'-deoxyuridine is abbreviated as ip^{5}dU. The ip^5 is also used in ip^{5}dUMP, ip^{5}dUTP and the copolymer poly(dA-dT,ip^{5}dU). UV, CD and EM stand for ultraviolet, circular dichroism and electron microscopy, respectively.

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molecules (3-6). This modified viral DNA was modelled earlier by poly(dA-dT) where dTMP could partially be replaced by ip^{5}dUMP (7). Structure of poly(dA-dT,ip^{5}dU) is characterized in the present study by EM, UV absorption and CD spectroscopies in order to elucidate contribution of conformation to the altered biochemical properties observed earlier (7). For this purpose poly(dA-dT,ip^{5}dU)-s containing 0, 1.3, 4.3 and 7.1 % ip^{5}dU were synthesized by *E. coli* Klenow DNA polymerase enzyme.

EXPERIMENTAL

Synthesis

Poly(dA-dT) and poly(dA-dT,ip^{5}dU)-s were prepared by methods applied earlier (7). Reaction mixtures contained 95%-5%, 75%-25% and 25%-75% of dTTP : ip^{5}dUTP to obtain poly(dA-dT,ip^{5}dU)-s with about 1%, 4% and the maximum amount (7-8%) of ip^{5}dUMP, based on the results of analytical reactions (7). In the 25 ml mixtures 80-120 OD_{max} units of high-molecular weight copolymers, excluded on Bio-Gel A-5m (Bio-Rad Labs) column, were formed.

Nucleoside compositions were determined from the enzymic hydrolysates of the polymers by HPLC (ISCO, Supersil 3 μm Si 4x100 mm column, EtAc-MeOH {98%-2%} eluent, 0.6 ml/min). Poly(dA-dT,ip^{5}dU)-s contained 1.3%, 4.3% and 7.1% ip^{5}dU, that is 2.6, 8.6 and 14.2% of dT were replaced by ip^{5}dU.

Heterogenity of the copolymers synthesized was determined by neutral and alkaline agarose (0.5 %) gel electrophoresis with 18 marker DNAs ranging from 468 to 48502 base pairs. Neutral distribution of lengths and the main band in brackets were as follows for poly(dA-dT) with 0, 1.3, 4.3 and 7.1% ip^{5}dU: >100,000 - 810 (10,500), 5,200 - 300 (1800), 9,300 - 290 (3,200) and 3,000 - 270 (740), respectively.

Methods

UV absorption-temperature melting profiles were determined essentially as described earlier (8), with a heating rate of 0.5 ^oC/min. Spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer.

CD measurements were carried out using a Jobin-Yvon dichrograph Mark IV calibrated with isoandrosterone. Concentrations of the polymers, calculated by using $\varepsilon(P) = 6640$ $M^{-1}cm^{-1}$ for poly(dA-dT) and the poly(dA-dT,ip^{5}dU)-s, ranged within 30-50 μg/ml.

The samples for EM analysis were taken from stock solutions used for CD measurements. Each polydeoxynucleotide was analysed by three techniques, namely modified aqueous, formamide and BAC, as described previously (9-11). Electron micrographs were recorded at magnification of 10,000 in a JEOL JEM 1200EX microscope operating at 60 kV, enlarged fivefold.

RESULTS

Poly(dA-dT) analogues were first studied by the absorbance *versus* temperature melting profiles. Figure 1 shows that only thermal transition of poly(dA-dT,ip^{5}dU) of 7.1% ip^{5}dU was unusual,

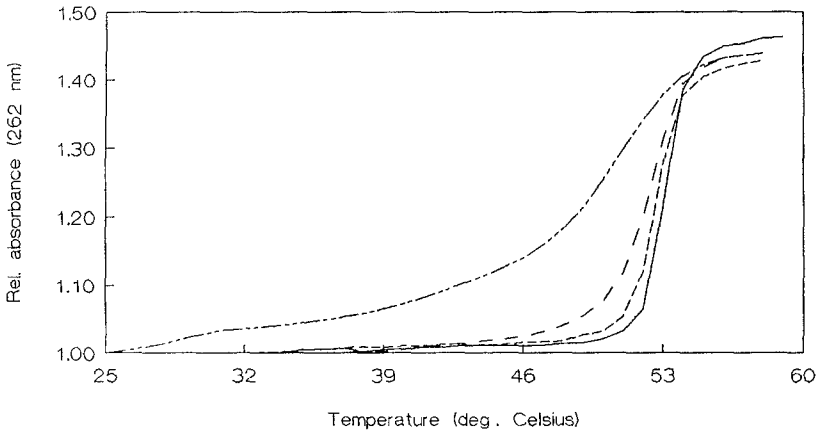

Figure 1. UV melting profiles of poly(dA-dT,ip^{5}dU)-s with 0%, —— ; 1.3%, – – – –; 4.3%, ———; and 7.1% ip^{5}dU, ——– – ——. Melting profiles were determined in 25 mM NaCl, 10 mM Tris.HCl (pH 7.2), 1 mM EDTA solution. Based on the first derivative of the transition curve, T_m values of 53.0, 52.9, 52.5 and 50.9°C were obtained for poly(dA-dT,ip^{5}dU)-s with 0, 1.3, 4.3 and 7.1% ip^{5}dU, respectively. T_m's were reproducible within ±0.2°C. Thermal hyperchromicities at 262 nm were between 43.1 and 45.8%. Widths of transitions, defined by the temperature range between 25 and 75% of the thermal hyperchromicity, were 1.2, 1.5, 2.3 and 7.6°C for polydeoxynucleotides of the above sequence.

its UV absorption at 260 nm increased gradually far before the main cooperative part of melting of the duplex. Substitution caused only small decrease in the stability of the duplexes.

CD spectrum of poly(dA-dT) exhibits temperature-induced changes prior to melting. In addition, denaturation is accompanied by a positive band red-shift (12). We observed similar changes with poly(dA-dT,ip^{5}dU) molecules containing 0, 1.3 or 4.3% ip^{5}dU (Figure 2, right panel). However, the positive band of poly(dA-dT,ip^{5}dU) of 7.1% ip^{5}dU-content showed a different temperature-dependence and the melting itself exhibited only a low degree of cooperativity. The red-shift of the positive band maximum was cooperative with poly(dA-dT)-s up to 4.3% ip^{5}dU but it turned into a gradual dependence where ip^{5}dU was 7.1% (Figure 2, panel right, bottom).

Further insight into the perturbations was obtained by EM, though the polydeoxynucleotides were inhomogeneous in length (see Experimental) that makes detailed statistical analysis of the EM data impossible. Three different EM techniques were used: the "aqueous" (9), the "formamide" (9,11) and the "BAC" spreading technique (10,11). The "aqueous" technique (9) is presented only

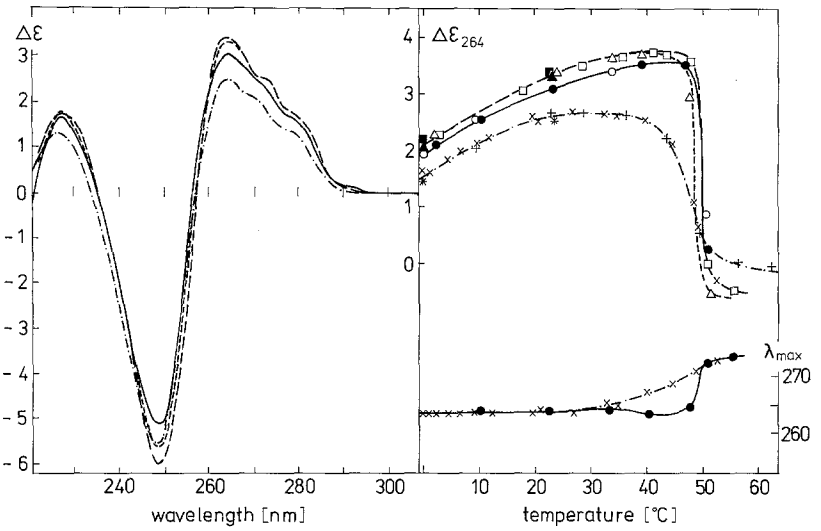

Figure 2.

Left panel: CD spectra of poly(dA-dT), ——— ; and poly(dA-dT,ip^{5}dU)'s with ip^{5}dU-content of 1.3%, — — — ; 4.3%, - - - ; and 7.1%, - · - · - ; determined in 25 mM NaCl, 10 mM Tris.HCl (pH 7.3), 1 mM EDTA at 23.2°C.
Right panel: Temperature-induced changes in the CD spectra monitored by ellipticity at 264 nm (top of right panel) and by the shift of the positive band maximum (bottom). Poly(dA-dT), ——— ; and analogues with ip^{5}dU-content of 1.3%, — — — ; 4.3%, - - - ; and 7.1%, - · - · - , respectively. Different symbols on the same curves, like o and ●, □ and ■, Δ and ▲, or x, + and * represent repeated determinations.

here which visualized poly(dA-dT) as filaments of variable lengths containing long branches (Figure 3 A). Each technique used showed that the introduction of ip^{5}dU into poly(dA-dT) made the macromolecules shorter and that the number of non-branched molecules decreased whereas the number of branches per molecule increased with the increasing ip^{5}dU content (Figures 3B-3D).

DISCUSSION

The crystal structure of ip^{5}dU shows that one of the two *iso*propyl methyls protrudes significantly (1.59 Å) from the attached base plane, making the formation of regular B-DNA impossible (13). However, the thermal melting profiles of poly(dA-dT,ip^{5}dU) with 4.3% or less ip^{5}dU were normal (Figures 1 and 2). In other words, if on average every 24th nucleotide in poly(dA-dT) is replaced by ip^{5}dUMP, poly(dA-dT) can buffer the perturbations introduced by the bulky *iso*propyl groups in the double helix major groove. Poly(dA-dT) is either different enough

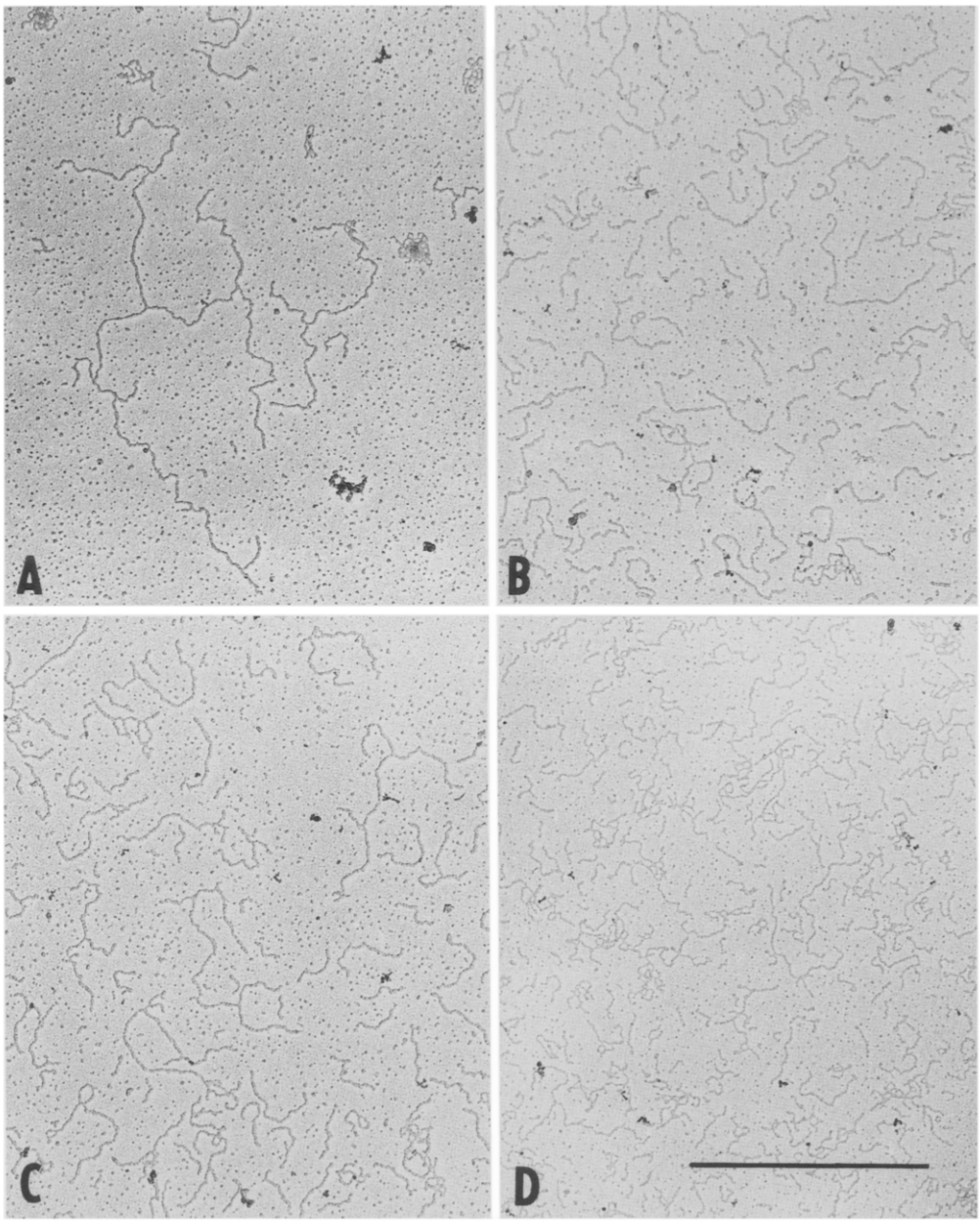

Figure 3.
Electron microscopic pictures of poly(dA-dT,ip^{5}dU)'s containing 0%, A; 1.3%, B; 4.3%, C; and 7.1% ip^{5}dU, D obtained by the "aqueous" technique. The bar is 1 μm.

from regular B-DNA (14,15) or flexible enough (16) to accomodate solitary ip^{5}dU-s.

If the replacement occurs with a higher frequency, i.e. each 14th nucleotide is replaced by ip^{5}dUMP (7.1% ip^{5}dU), the melting

profiles become qualitatively different. This can be explained by independent melting out of small molecule parts before the melting of the whole molecule takes place. Incorporation of ip^5dU is probably not uniform. The DNA strands where several dA-ip^5dU dimers follow each other, even if DNA polymerase could synthetize it, the duplex can no longer tolerate their presence. Sequential irregularities rather tend to bulge out and form hairpins where ip^5dU-s are assumed to be in the loop. These hairpins seem to be stable as compared to hairpin structures in poly(dA-dT,ip^5dU)-s with 4.3% and lower ip^5dU-content. The latter stem-loops, visualized only by EM, are metastable in solution and thus are not shown by the T_m-curves. So far, hairpins have been found to be stable in response to negative supercoiling in circular DNA molecules (17,18), in artificial linear DNA constructs (12) and in oligodeoxynucleotide structures (e.g. 19) or in RNAs (20).

Poly(dA-dT,ip^5dU) molecules become shorter as the ip^5dU-content increases. Presumably the polymerase enzyme dissociates more frequently from the distorted replication complex than from poly(dA-dT). Shortening is shown by EM (Figure 3) and agarose gel electrophoresis (Experimental). EM also clearly shows that the number of branches increases in poly(dA-dT) with the amount of ip^5dU. Thus, incorporated ip^5dU induces branching. Branches in poly(dA-dT) are long-stem stem-loops structures. Up to 4.3% ip^5dU-content stem-loops are metastable in poly(dA-dT,ip^5dU) and can be seen only on the EM pictures. Spectroscopy shows only the more stable hairpin-loop structures induced by 7.1% ip^5dU.

The presence of stable hairpins in poly(dA-dT,ip^5dU) containing 7.1% ip^5dU is well compatible with its strongly enhanced sensitivity to the single-strand-specific nuclease S1, as compared to poly(dA-dT) (7). The increased sensitivity can be explained by the presence of real single-stranded regions in poly(dA-dT,ip^5dU), occuring in the hairpin loops, similarly as observed with oligodeoxynucleotide loop structures (19). In this way, herpes-virus DNA synthesized *in vivo* in the presence of the antiherpetic ip^5dU may be destabilized against some cellular endonucleases if a sufficient level of substitution by ip^5dUMP for dTMP is reached.

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