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INHIBITION OF c-H-RAS ONCOGENE INDUCED TRANSFORMATION OF RAT EMBRYO FIBROBLASTS BY COTRANSFECTED POLYNUCLEOTIDES CONTAINING ALTERNATING PURINE-PYRIMIDINE SEQUENCES

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SUMMARY: When Rat 6 cultures were cotransfected with an activated c-H-ras oncogene (pT24) and poly(dG-m^5dC), a synthetic polymer that has the potential to form Z DNA, there was marked inhibition of cell transformation. Cotransfection of pT24 DNA with poly(dG-dC) caused somewhat less inhibition, poly(dA-dC).(dG-dT) caused moderate inhibition, and poly(dG).(dC) exerted negligible inhibition. Evidence was obtained that the inhibition seen with poly(dG-m^5dC) was not simply due to an inhibition of cellular uptake of the pT24 DNA. Our results suggest that certain polymers that have the potential to form Z DNA can inhibit the integration and expression of a transfected oncogene.

The discovery that certain alternating purine and pyrimidine DNA sequences assume a left-handed Z-DNA structure, under specific conditions, represents an important development in the biochemistry of DNA (1). Potential Z-DNA forming regions have been detected in the chromosomes of various species by using Z-DNA specific antibodies (2,3). Since it appears that a close relationship exists between DNA conformation and gene replication and expression (4), Z-DNA may play an important biologic role. We have recently reported that certain alternating purine-pyrimidine sequences with the potential to form a left-handed Z-DNA, when cotransfected with the thymidine kinase (tk) gene, decreased the frequency of tk^+ transformed colonies in Ltk^- cells (5). On the other hand, the same polymers enhanced the transient expression of the chloramphenicol acetyltransferase gene in these cells (6). In an effort to learn more about the possible biological role of polymers with the potential to form the Z-conformation it was of interest to study the effects of these

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polymers on the transformation of cells by an activated oncogene. In recent studies (7) we found that the Rat 6 fibroblast cell line shows a low frequency of transformed foci when transfected with an activated human c-H-ras bladder cancer oncogene (T24), but this frequency can be markedly enhanced when the cell are grown in the presence of a phorbol ester tumor promoter or fetal calf serum (FCS) (7, 8). Therefore, this transformation system provides a useful model to evaluate other agents which might affect cell transformation.

MATERIALS AND METHODS

Cell Cultures and Growth Medium: The Rat 6 cells were subcloned from an established F2048 rat embryo fibroblast culture originally isolated by Freeman and his colleagues (9). The stock cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Flow Laboratories, McLean, Va.). Cultures were maintained in a humidified incubator at 37°C with 5% CO_2 in air and fed twice a week with fresh medium.

Plasmid and synthetic polydeoxyribonucleotides: Plasmid pT24 containing a 6.4 kilobase (kb) BamHI fragment corresponding to the cellular coding sequence of the mutated human bladder cancer c-H-ras oncogene (10) was obtained from M. Wigler. All of the double-stranded polymers were obtained from Pharmacia P-L Biochemicals.

Transfection assays: DNA-calcium phosphate mediated transfection procedure was performed as previously described (11, 12). Briefly, Rat 6 cells were seeded at 5 X 10^5 per 90 mm plate in DMEM plus 10% calf serum (D10) about 24 hrs before transfection. They were fed with fresh medium 4 hrs prior to transfection with the DNA-calcium phosphate precipitate, which consisted of 1 µg of pT24 DNA, various amounts of the polymers, as indicated in the specific experiments, and sufficient rat genomic DNA to bring the total amount of DNA up to 20 µg. Approximately two to three weeks following transfection the plates were stained with Giemsa and the number of transformed foci counted, as previously described (7, 8).

Isolation of Nuclear DNA: Rat 6 cells were transfected with T24 plasmid DNA with or without the indicated polymers, as described above. The cells were harvested 24, 48, 72 or 144 hrs later. Nuclei were isolated essentially by the method of Dignam et al (13). To isolate DNA, the nuclei were incubated with 1.5ml of lysis buffer (10mM Tris HCl, pH 7.9, 10mM EDTA. 10mM NaCl, 0.1% sodium dodecyl sulfate) and 0.2 mg per ml of proteinase K (Boehringer- Mannheim) for 1.5 hrs. at 37°C. The lysate was extracted successively with Tris buffer (pH 7.8) saturated phenol, phenol: chloroform plus 4% iso-amyl alcohol (1:1) and chloroform iso-amyl alcohol (24:1) and the DNA was ethanol precipitated in the presence of 0.2M sodium acetate at -20°C overnight.

Southern Blot Analyses of DNA: Restriction enzyme digestion, gel electrophoreses and Southern blot hybridizations (14) of DNA samples, and the preparation of a ^{32}P-labelled probe by nick translation of a 3Kb T24 specific SacI fragment isolated from pT24 plasmid DNA, were performed as previously described (5).

RESULTS

Effects of various polynucleotides on the transformation of Rat 6 cells when cotransfected with the pT24 oncogene.

In order to evaluate the effects of various polymers on oncogene-induced transformation of Rat 6 fibroblast cells, we cotransfected pT24 DNA together with a variety of polynucleotides. Following transfection, the cells were grown in the presence of 5% FCS to enhance the frequency of cell transformation (8). Figure 1 shows the effects of increasing concentrations of the various polymers on the oncogene-induced transformation of Rat 6 cells. In the absence of polymer, the control plate gave rise to 175 T24 induced foci per 5 x 10^5 recipient cells. Two polymers which are known to readily form Z-DNA, poly(dG-m^5dC) and poly(dG-dC) displayed a concentration-dependent inhibition of oncogene-induced transformation. Inhibition was also seen with poly(dA-dC).(dG-dT), which can form Z-DNA under certain conditions (1), but this polymer was less potent. It is of interest that 4 µg per plate of the alternating polymer poly(dA-dT), which has been shown recently to be able to form Z-DNA (17), also produced about a 60% inhibition of transformation (not

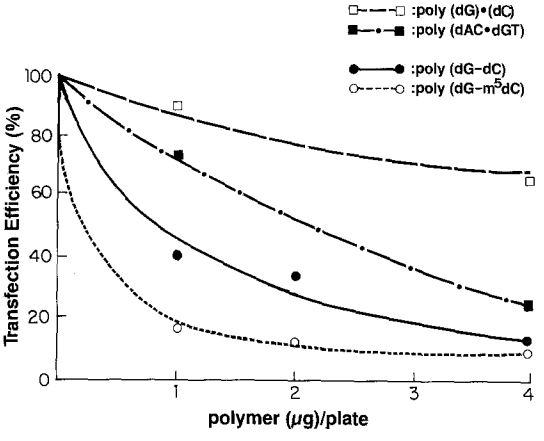

Figure 1: Effects of increasing concentrations of various polymers on cell transformation when they are cotransfected with 1 µg of pT24 DNA onto Rat 6 cells. The total number of transformed foci in the control plate was 175 foci per 5x10^5 recipient cells, and this value was considered as 100%. Separate studies indicated that maximum transformation was obtained with 1 µg of pT24 DNA.

Table 1. Effects of poly(dG-m^5dC) on T24-induced transformation under various conditions

Group	Transfected DNAs	Condition of poly(dG-m^5dC)	Transformation (1% of Control)
A (control)	pT24		100%
B	pT24 + poly(dG-m^5dC)	Ca^{++}ppt in same tube as pT24, cotransfected at the same time	10%
C	pT24 + poly(dG-m^5dC)	Ca^{++}ppt in a separate tube, transfected at the same time as pT24	29%
D	pT24	soluble poly(dG-m^5dC) was added to the cultures during each feedings	90%
E (control)	pT24	Rat 6 DNA was transfected 2 days after the pT24 transfection	100%
F	pT24	poly(dG-m^5dC) was transfected 2 days after the pT24 transfection	197%

Except for the indicated changes the procedure was essentially the same as that described in Figure 1. Assays contained 1 µg of pT24 DNA and, where indicated, 4 µg of poly(dG-m^5dC). In Group E 4 µg of Rat 6 genomic DNA was substituted for the polymer. The absolute value of transformed foci in the control cultures A and E was about 300 and 100 foci, per 5 X 10^5 cells, respectively, and these values were considered as 100%.

shown). On the other hand, the homopolymer poly(dG).(dC), which does not form Z-DNA, produced only a slight inhibition of cell transformation. Thus, the inhibition observed with the previous polymers is not due to nonspecific effects.

Since poly(dG-m^5dC) was the most potent inhibitor (Figure 1), and since among the various polymers tested it also most readily adopts the Z-conformation (1), additional studies were done with this polymer. An inhibitory effect was seen when the pT24 DNA and the polymer were precipitated with calcium phosphate in separate tubes and then added simultaneously to the culture dishes, but this inhibition was three fold less than that obtained when the pT24 DNA and polymer were coprecipitated (Table 1, group C vs group B). Poly(dG-m^5dC) did not exert an inhibitory

effect on T24-focus formation if it was transfected into Rat 6 cells two days after the transfection of pT24 DNA into the same cultures (group F vs E. Table 1). Control plates in group E were transfected with genomic carrier DNA instead of poly(dG-m^5dC) for the second round of transfection. Simple addition of soluble poly(dG-m^5dC) into the culture medium, following transfection of pT24 DNAs, did not significantly affect transformation frequencies (Table 1, group D). Taken together, these findings indicate that poly(dG-m^5dC) exerts its maximum inhibition when coprecipitated and cotransfected with the pT24 DNA, suggesting that it exerts its effects at an early stage in the transfection process.

Effects of poly(dG-m^5dC) on DNA uptake and DNA integration in Rat 6 cells.

In view of the above results, it was possible that the polymer acted by inhibiting cellular uptake of the pT24 DNA. To investigate this possibility, we cotransfected Rat 6 cells with either 1 μg of pT24 DNA plus 4 μg of poly(dG-m^5dC) and 15 μg carrier DNA, or with 1 μg of pT24 DNA with 19 μg of carrier DNA, under the same conditions as described above. We then isolated total nuclear DNA from the Rat 6 cells, at 24, 48, 72 or 144 hrs after the transfection procedure. Samples of undigested, BamHI digested or SacI digested DNAs were separated by agarose gel electrophoresis and analysed by Southern blot hybridization using a T24-specific ^{32}P-labeled probe. Figure 2 indicates that at 48 hr the total level of T24 DNA appeared to be actually less in the cells transfected with T24 alone (lane 1-3) than in the cells transfected with T24 plus poly(dG-m^5dC) (lanes 4-6). It is remarkable that even at 72 hrs the control DNA samples showed a somewhat lower level of T24 DNA (Figure 2, lanes 7-9) than the DNA samples from the cells cotransfected with poly(dG-m^5dC) (lanes 10-12). The 24 and 144 hour time points also showed a higher level of T24 DNA in the cells cotransfected with the polymer (data not shown). It appears, therefore, that cotransfection with poly(dG-m^5dC) may enhance rather than inhibit cellular uptake (or retention) of T24 DNA.

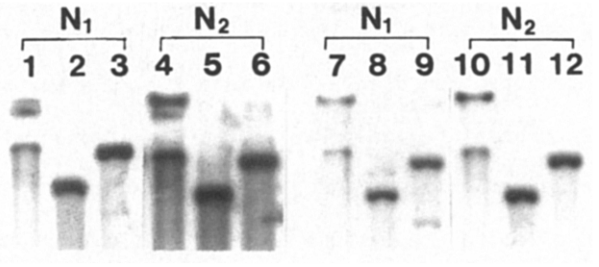

Figure 2: Southern blot analyses of nuclear DNAs extracted from Rat 6 cells at 48 (lanes 1-6) and 72 hrs (lanes 7-12) after transfection with pT24 or with pT24 DNA and poly(dG-m^5dC), using a ^{32}P-labeled pT24 DNA as the probe. N_1 - nuclear DNA from cells transfected with pT24 DNA (1 µg); N_2 - nuclear DNA from Rat 6 cells cotransfected with pT24 DNA (1 µg) and poly(dG-m^5dC), (4µg). Lanes 1,4,7,10 - uncut DNAs; Lanes 2,5,8.11 - BamH1 restricted DNAs; Lanes 3,6,9,12 - SacI restricted DNAs. Five µg of the indicated DNA were applied to each lane.

DISCUSSION

The present results indicate that certain polymers with the potential to form Z-DNA inhibit the oncogenic transformation of Rat 6 cells by an activated human bladder cancer c-H-*ras* oncogene when they are cotransfected with a plasmid (pT24) containing the latter oncogene DNA sequence. The polymer with the strongest potential to adopt the Z-conformation, poly(dG-m^5dC), had the strongest inhibitory effect. On the other hand, a homopolymer poly(dG).(dC) that does not form Z DNA (1) exerted only a slight inhibition (Figure 1). Although it is tempting to assume that the effects we have observed are due to the ability of certain polymers to adopt the Z-DNA conformation we should stress that we do not know that these polymers adopt this conformation under our experimental conditions.

Although the present studies do not reveal the precise mechanism by which a polymer like poly(dG-m^5dC) inhibits T24-induced cell transformation, we have obtained evidence that this polymer does not act simply by blocking the cellular uptake of the T24 DNA (Figure 2). Since there is evidence that DNA sequences that can adopt the Z conformation can influence DNA recombination (16), it is possible that in our cotransfection studies such polymers complex with and tie up cellular

proteins involved in DNA recombination, and thus inhibit integration of the T24 DNA into the genome of the host cell. Obviously, further studies are required to evaluate these and other possible explanations. Nevertheless, the system described in the present study may be valuable for elucidating specific biologic functions of unusual DNA sequences that can form Z-DNA or other non-B-DNA conformations.

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