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THE PREPARATION OF POLY (dT)-5′-TRANSFERRIN CONJUGATES AND HYBRIDISATION STUDIES WITH POLY (dA)-TAILED LINEARISED pBR322 PLASMID DNA

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Abstract—The formation of transferrin–DNA complexes intended for ligand-directed transfection studies has been achieved through a hybridisation technique involving complementary homodeoxypolynucleotide chains attached to the participating protein and DNA species. Oligothymidylate residues $(pT)_n$ obtained by dicyclohexylcarbodiimide (CDI) polymerisation of thymidine-5′-monophosphate (5′-TMP) were activated to the 5′-imidazolides which on incubation with transferrin yielded the 5′linked phosphoramidates $(pT)_n$-5′-transferrin. Homopolymeric chain extension of $(pT)_5$-5′-transferrin by terminal transferase and dTTP at 30° for 30 min yielded $(pT)_{300}$-5′-transferrin. Cleavage of the phosphoramide link in the polymer modified transferrin at 37° was pronounced after 30 min although at 25° hydrolysis was <5% after 4 hr. Poly(dT)-5′-transferrin readily hybridised with [^{3}H]poly(dA)-tailed Pst 1 linearised pBR322 DNA. Resultant complexes were demonstrated by nitrocellulose filter binding and immunoprecipitation with anti-transferrin antibody. In contrast with poly(dT)-5′-transferrin, poly(dT)-5′-transferrin-poly(dA)-tailed pBR322 DNA complexes were stable at 37° suggesting that annealing is followed by further stabilising interactions between the DNA and protein components.

It has been suggested by Pastan and co-workers that corrective DNA may be introduced into genetically defective cells of intact organisms by attaching the DNA to protein ligands which may be recognized by cognate plasma membrane receptors on the target cell [1]. The protein–DNA complex would then be internalized by receptor mediated endocytosis. This concept of ligand directed transfection is currently under investigation in our laboratory. Central to our approach is the need to construct stable "non-covalent" protein–DNA complexes in which chemical modification of the DNA is minimal or absent. In this regard double stranded DNA lends itself to two types of association: (i) electrostatic attraction involving the phosphodiester backbone, and (ii) hydrophobic interaction of the bases.

We have demonstrated the type (i) association with albumin and the ligand transferrin [2] by treating these proteins with the water soluble carbodiimides *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (CDI)† and *N*-ethyl-*N*′-(trimethylpropylammonium) carbodiimide iodide (Me^+CDI) under controlled conditions which lead to the formation of basic *N*-acyl urea moieties at glutamate and aspartate residues [3]. The *N*-acylurea proteins form cohesive dissociable salt bridges with ionized phosphate residues of the DNA [2]. Related studies with other peptide and protein ligands are in progress.

Type (ii) association has been studied using lipophilic derivatives of the intercalating dye ethidium bromide. Hence 3- and 8-cholesteryl succinamido ethidium provide the structural features required to anchor DNA to lipid surfaces [4, 5]. Derivatives such as these may also be useful in enhancing DNA complex formation with liposomes (manuscript in preparation).

We report here on the application of a third type of interaction which is possible with single stranded DNA. Thus linear duplex DNA may be tailed homopolymerically and anchored to a protein ligand by specific hydrogen bonding to protein-bound complementary deoxyoligonucleotide fragments as shown in Fig. 1. The thymidine pentamer $(pT)_5$ was immobilized to transferrin via a phosphoramide link involving the oligomer 5′-phosphate group (Fig. 1). The orientation of the $(pT)_5$ chains permitted the subsequent enzymatic extension of the oligomers at the 3′-termini to $(pT)_{300}$ using terminal transferase with thymidine-5′-triphosphate (dTTP). This measure was taken to improve T_m values of transferrin-DNA complexes formed on hybridization with poly(dA)-tailed pBR322 DNA.

MATERIALS AND METHODS

Materials. Human serum transferrin, bovine serum albumin, dATP, dTTP, dTMP and CTP were from the Sigma Chemical Co. (St Louis, MO). Terminal deoxynucleotidyl transferase (EC 2.7.7.31) and decathymidylic acid were obtained from P.L. Biochemicals Inc. (Milwaukee, WI). Restriction endonuclease Pst 1 (EC 3.1.23.31) and pBR322 DNA were from Boehringer-Mannheim (Hamburg,

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† Abbreviations used: CDI, *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide; Me^+CDI, *N*-ethyl-*N*′-(3-trimethylpropylammonium) carbodiimide iodide; DCC, dicyclohexylcarbodiimide; TEAB, triethylammonium bicarbonate.

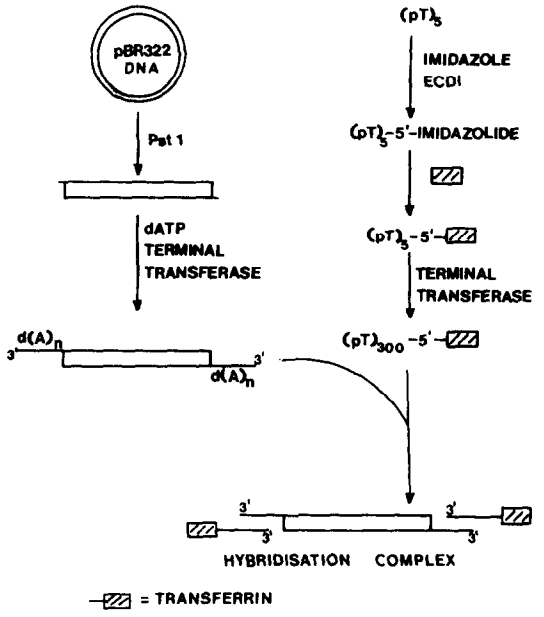

Fig. 1. Outline of experimental procedure in the construction of poly (dT)-transferrin/poly (dA)-tailed pBR322 DNA complexes.

F.R.G.). [^{3}H]dTTP (97 Ci/mmol), [^{3}H]dTMP (60 Ci/mmol), [^{3}H]CTP (20 Ci/mmol) and [^{3}H]dATP (26 Ci/mmol) were obtained from Amersham U.K. *N*-Ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide HCl (CDI), dicyclohexylcarbodiimide (DCC), phosphodiesterase 1 and $60F_{254}$ TLC plates were from Merck (Darmstadt, F.R.G.). Agarose (ultra pure grade), acrylamide and bisacrylamide were obtained from BioRad. DEAE Sephacel, Sephadex G-50 (superfine) and G-100 were obtained from Pharmacia, Uppsala, Sweden. Goat anti human transferrin antibody was from Bio-Yeda, Rehovat, Israel. All other reagents were of analytical grade.

Thymidine oligomers. Polymerisation of thymidine-5'-monophosphate (dTMP) was carried out with DCC in dry pyridine by the method of Khorana and Vizsolyi [6]. Oligomeric products of up to eight units in length, $(pT)_8$, were purified by HPLC on a preparative aminohexyl column (Zorbax-NH_2, 21.2 mm × 25 cm) using a linear gradient of 30% acetonitrile: 70% 0.05 M triethylammonium bicarbonate (TEAB, pH 7.6) to 100% 0.5 M TEAB (pH 7.6) over 30 min at a flow rate of 24 ml/min. The purified products were collected by hand and freeze dried. Samples were analysed by TLC on silica gel $60F_{254}$ plates in ethanol: 0.5 M ammonium acetate (pH 3.8) [7:2 v/v]. Products were quantitated by UV using $E_{271} = 9.7 \times 10^3$ (pH 1.5–7.0) for thymidine [7]. No correction was made for hyperchromicity.

Chain length determination of thymidine oligomers. Samples (100–200 μg) of each oligomer fraction were incubated with snake venom phosphodiesterase (5 μg) in 50 μl incubation buffer containing 20 mM Tris–HCl (pH 7.9), 2 mM Mg $(OAc)_2$. Aliquots (5 × 10 μl) were withdrawn from digests at regular intervals up to 7 min from digests of fractions suspected to contain smaller oligomers ($\leq (pT)_4$) and cyclic products and dispensed directly into 50 μl of a stop solution containing 2 mM EDTA, 0.1 M NH_4OH. Partial digestion of larger oligomers was carried out over a longer period (30 min; 7 × 7 μl aliquots). Partial digests were analysed on an ODS HPLC column (Partisil ODS, 4.6 mm × 25 cm from Philips) using a linear gradient from 100% 0.1 M ammonium acetate (pH 5.8) to 30% acetonitrile: 70% 0.1 M ammonium acetate (pH 5.8). Eluates were monitored at 254 nm.

Preparation of [^{3}H] $(pT)_6C$ *and* [^{3}H] $(pT)_{10}C$. The method was adapted from that of Roychoudhury *et al.* [8]. Specific activities: [^{3}H] $(pT)_6C$, 4.1×10^4 cpm/nmole $(pT)_6$; [^{3}H] $(pT)_{10}C$, 2.6×10^4 cpm/nmole $(pT)_{10}$.

$(pT)_n5'$ *and* [^{3}H]$(pT)_n$ C-5'*-imidazolidates.* A general method was employed in the synthesis of 5'-imidazolidates which is exemplified by the following preparation: The thymidine pentamer $(pT)_5$ (3.12 μmole) was incubated in a solution (0.5 ml) containing ECDI (0.3 M) and imidazole (0.3 M, pH 6.0) for 1 hr at room temperature. The reaction mixture was applied to a Whatman 3MM paper (50 × 14 cm) and electrophoresed at 200 V (50 mA) for 45 min in 0.05 M TEAB (pH 7.6). The strongly UV_{254} absorbing band was extracted with 0.02 M TEAB (pH 7.6) (5 × 4 ml) and freeze dried. Yield of nucleotide material: 2.28 μmole (using $E_{271} = 9.7 \times 10^3$ for thymidine without correction [7]). Analysis by TLC on silica gel $60F_{254}$ analytical plates developed in isopropanol: ammonia:H_2O (7:1:2, v/v) gave $R_f = 0.43$ ($(pT)_5 = 0.16$).

Preparation of $(pT)_5$-5'*-transferrin.* $(pT)_5$-5'-imidazolidate (2.20 μmole) and transferrin (100 μg, 13 nmole) were incubated in 0.25 M collidine–HCl (150 μl, pH 7.5) for 14 hr at room temperature. The transferrin–oligomer conjugate was obtained by gel filtration of the reaction mixture on a Sephadex G-100 column (108 × 0.7 cm; 0.05 M TEAB pH 7.6; 6.5 ml/hour). Fractions (1.3 ml) were collected and monitored at 260 and 280 mm. The pooled protein fractions exhibited λ_{max} at 270 nm (cf. transferrin 280 nm).

Pst 1 *Digestion of* pBR322 *DNA.* pBR322 DNA (6.6 pmol) was digested with the restriction endonuclease Pst 1 (70 U) in 0.2 ml of a buffer containing 0.01 M Tris–HCl (pH 7.2), 0.05 M NaCl. 0.01 M $MgCl_2$ at 37° for 2 hr. The reaction mixture was concentrated to 80 μl under a stream of N_2 and stored at −20°. The linearised plasmid was purified by 1.2% agarose gel electrophoresis in a buffer containing 0.036 M Tris–HCl, 0.03 M sodium phosphate and 0.01 M EDTA (pH 7.6) followed by electroelution and DEAE sephacel ion exchange chromatography as described by Maniatis *et al.* [9]. Plasmid DNA was precipitated with cold ethanol (2 vol.) and pelletted by ultracentrifugation at 0° in a polyallomer tube (Beckman FA 40 rotor, 30 000 rpm, 30 min). The pellet was dissolved in H_2O (3 × 200 ml) and finally freeze dried. Yield: 3.5 μg.

Poly (dA) tailing of Pst 1 *digested* pBR322 *DNA.* To a solution of Pst 1 digested pBR322 DNA (0.65 μg, 0.22 pmole) and [^{3}H] dATP (14.8 nmole, 125 μCi) in a buffer (50 μl) containing 0.1 M pot-

Table 1. Coupling of 5′-imidazolidate derivatives of TMP and thymidine oligomers to transferrin

Imidazolidate	Transferrin conc. (mM)	Oligomer: Protein mole ratio	
		Reactants	Conjugates
[^{3}H]Im-TMP	0.005	1:1	0.01:1
[^{3}H]Im-TMP	0.005	50:1	0.05:1
[^{3}H]Im-(pT)$_6$C	2.5	1:1	0.01:1
[^{3}H]Im-(pT)$_{10}$C	0.009	20:1	0.01:1
Im-(pT)$_5$	0.008	1600:1	0.1:1*

Conjugate mole ratios were determined by a nitrocellulose filter binding assay.
* Im (pT)$_5$ was non radioactive and the conjugate mole ratio was inferred from [^{3}H]poly(dT)-transferrin.

assium cacodylate, 1 mM $CoCl_2$ and 0.2 mM dithioerythritol (pH 7.0) was added terminal transferase (50 U) and the mixture incubated for 1 hr at 37°. This was extracted with chloroform: phenol (1:1 v/w, 40 μl) and the aqueous layer applied to a Sephadex G-100 column (50 × 0.4 cm). Equilibration and elution was carried out with a buffer containing 10 mM Tris–HCl, 1 mM EDTA and 0.1 M NaCl (pH 7.6) at a flow rate of 5 ml per hour. The product was found to have a specific activity of 5.7×10^5 cpm per μg DNA corresponding to poly (dA) tail lengths of approximately 105 dA residues.

Polyacrylamide gel electrophoresis of polynucleotides. Samples (0.5 μg) were dissolved in TBE buffer containing 90% formamide before electrophoresis on 12% polyacrylamide gels containing 7M urea in TBE buffer. Gels were visualized by the silver staining procedure [10].

Nitrocellulose filter binding assay. The addition of [^{3}H]TMP to (pT)$_5$-5′-transferrin and the hybridisation of poly(dT)-transferrin to [^{3}H] poly(dA)-tailed pBR322 DNA was determined by filtering aliquots of reaction mixtures through Millipore, type HA, 0.45 μm filters presoaked in appropriate reaction buffers. Filters were washed with PBS (3 ml), and oven dried (80° for 20 min) and counted in scintillation fluid.

RESULTS

Preparative and analytical (pT)$_n$-5′- *and* (pT)$_n$C-5′-transferrin conjugates

Findings presented in Table 1 clearly show that the 5′-imidazolidates of TMP and oligo(dT) do not readily react with transferrin and the desired phosphoramidates are generated in low yield. The preparation of (pT)$_5$-5′-transferrin, the precursor of poly (dT)-transferrin, was therefore conducted at a high (pT)$_5$-5′-imidazolidate: transferrin mole ratio.

Characterization of poly(dT)-transferrin

Gel exclusion chromatography of [^{3}H]poly(dT)-transferrin is shown in Fig. 2a. The poly(dT) transferrin conjugate eluted ahead of the carrier transferrin indicating a larger molecular species. Presence of the polynucleotide in the conjugate was indicated by a bathochromic shift in the ultraviolet spectrum of fraction 9. Thus λ_{max} for fractions 9 and 11 were 269 and 280 nm respectively. The product containing fraction was further analysed by immunoprecipitation as follows: A 50 μl aliquot of the poly(dT)-transferrin fraction (1500 cpm) was mixed with carrier transferrin (60 μl, 0.2 μg/μl in PBS) and incubated with goat anti-human transferrin antibody (43 μl, 3.5 μg antibody/μl) at 10° for 2 hr. The precipitate was pelletted (9000 *g*, 2 min) and the radioactivity associated with the supernatant and pellet (transferrin) was determined by liquid scintillation. (280 and 930 cpm respectively).

The chain length of transferrin linked poly (dT) was estimated by hydrolytic cleavage of the phosphoramide links in the conjugate in fraction 9 followed by polyacrylamide gel electrophoresis of the digest under denaturing conditions against molecular weight markers. As shown in Fig. 2b most fragments were circa 300 nucleotides in length. Using this value and the specific activity of incorporated [^{3}H] TMP residues (20,000 cpm/nmole) the mole ratio of poly(dT) to transferrin in poly(dT)-transferrin was estimated to be 0.1:1.

It was noted that the phosphoramide bond linking the primer oligo (dT) chains to transferrin underwent significant hydrolysis during terminal transferase catalysed chain extension. This was observed as a marked decrease in nitrocellulose filter retained radioactivity after 30 minutes of incubation at 37° (Fig. 3). The subsequent increase in radioactivity retained on filters after 1 hr of incubation and thereafter is attributed to free poly(dT) chains which have reached the minimum threshold length necessary for nitrocellulose filter binding. In a separate experiment chain extension was terminated with EDTA after 30 min at 37° and the mixture maintained at 25°. An assay after 4 hr showed minimal hydrolysis. Reincubation at 37°, however, led to marked cleavage of nucleo-protein conjugate (Fig. 3).

Hybridisation of [^{3}H]*poly(dT)-transferrin to* [^{3}H] *poly (dA)-tailed* pBR322 *DNA*

Figure 4 shows the hybridisation of varying concentrations of [^{3}H] poly(dT)-transferrin to [^{3}H]poly(dA)-tailed pBR322 DNA as determined by a nitrocellulose filter binding assay [11–13]. Saturation is reached at a mole ratio of about 2:1 as expected from theoretical considerations. The low

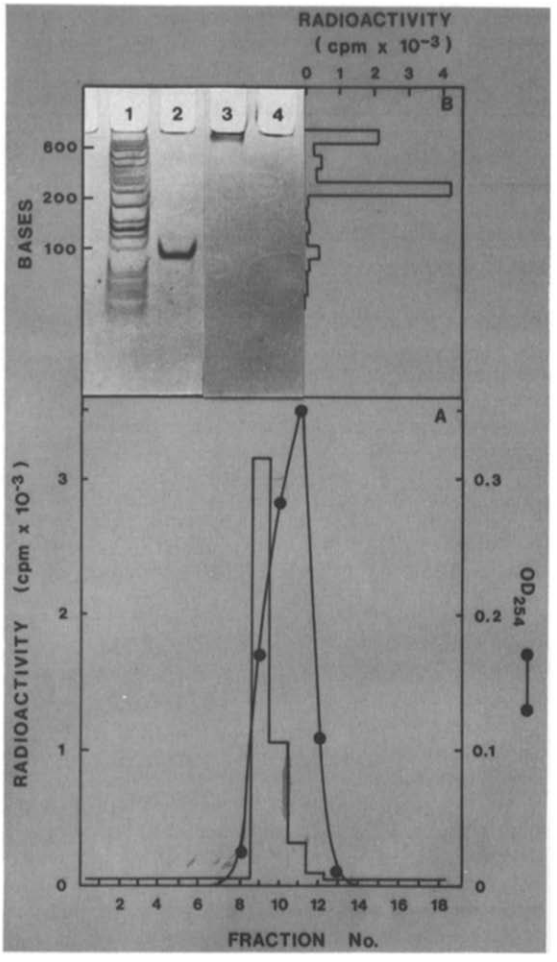

Fig. 2. Isolation to [^{3}H] poly(dT)-transferrin and [^{3}H] poly(dT) chain length estimation. (A) To a reaction mixture (280 μl) containing $(pT)_5$-5'-transferrin (0.5 nmole w.r. to transferrin), [^{3}H]TTP (2 μmole, 40 μCi), terminal transferase (60 U), 0.1 M potassium cacodylate, 1 mM $CoCl_2$ and 0.2 mM dithioerythritol (pH 7.0) was added EDTA (12 μl, 0.1 M) and transferrin carrier (100 μg) after 30 min at 37°. Poly(dT)-Transferrin was obtained by gel exclusion (Sephadex G-100, column: 108 × 0.7 cm,) using TEAB 0.05 M (pH 7.6) as eluant (1.3 ml fractions). Aliquots (100 μl) of each fraction were taken for determination of radioactivity. (B) An aliquot (100 μl) of fraction 9 was heated at 60°/3 hr before electrophoresis (lane 4) on 12% polyacrylamide in TBE buffer against 1, Alu 1 digest of pBR322 DNA (0.4 μg); 2, tRNA (50 ng) and 3, transferrin (5 μg). Gel slices (2 mm) were taken for digestion followed by radioactivity determination in scintillation fluid.

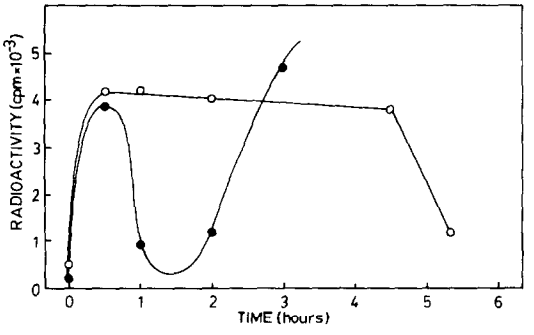

Fig. 3. Addition of [^{3}H]TMP residues onto oligomer of $(pT)_5$-5'-transferrin. $(pT)_5$-5'-Transferrin (125 pmole) was incubated at 37° with [^{3}H]TTP (500 nmole, 10 μCi) and terminal transferase (10 U) in 70 μl incubation buffer (legend to Fig. 2). Aliquots (14 μl) were removed at regular intervals and assayed (●—●). In a separate experiment chain elongation was terminated after 30 min at 37° by addition of EDTA (4 mM final conc). The temperature was lowered to 25° and restored to 37° after 4 hr (○—○). Assays were by nitrocellulose filter binding.

Effect of temperature on poly(dT)-transferrin complexes with poly (dA)-tailed linearised pBR322 *DNA*

In light of the demonstrable temperature sensitivity of the phosphoramide bond in poly(dT)-transferrin (Fig. 4) it was considered necessary to investigate the stability of poly(dT)-transferrin poly(dA)-pBR322 DNA complexes. [^{3}H] poly(dA)-tailed linear pBR322 DNA (2 pmol, 2400 cpm) was incubated with poly (dT)-transferrin (0.25 μg w.r.t. transferrin) in PBS (80 μl) at 5° for 1 hr. The temperature was then raised to 37° and samples (13 μl) were removed at $T = 0$, 2, 5, 10, 20 and 40 min and analysed by a nitrocellulose filter binding assay. The results obtained (860, 846, 901, 783, 940 and 957 cpm respectively) indicate a marked stability of the

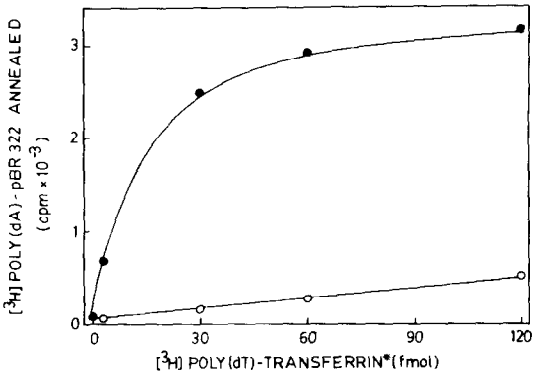

Fig. 4. Annealing of poly(dT)-transferrin to poly (dA)-tailed pBR322 DNA. Incubation mixtures contained [^{3}H] poly (dA)-tailed pBR322 DNA (6 fmole, 12000 cpm) and varying amounts of [^{3}H] poly(dT)-transferrin as indicated, in 60 μl of PBS. After 2 hr at 10° 2 μg of transferrin was added to each reaction mixture and hybridization was assessed by nitrocellulose filter binding (●—●). Radioactivity contributed by [^{3}H] poly(dT)-transferrin alone was determined in the absence of [^{3}H] poly(dA)-tailed pBR322 DNA (○—○). * Concentration with respect to poly(dT).

levels of radioactivity contributed by [^{3}H] poly(dT)-transferrin to the filter bound complexes were determined in control experiments lacking the [^{3}H] poly (dA) tailed linearised plasmid DNA and are shown in Fig. 4.

Hybridisation complexes were also demonstrated by immunoprecipitation as illustrated in Fig. 5. Hence in control experiments [^{3}H] poly(dA)-tailed pBR322 DNA was not associated with immunoprecipitates of transferrin whilst the poly (dA)-tailed linearised plasmid DNA was clearly bound to the immunoprecipitated poly (dT)-transferrin.

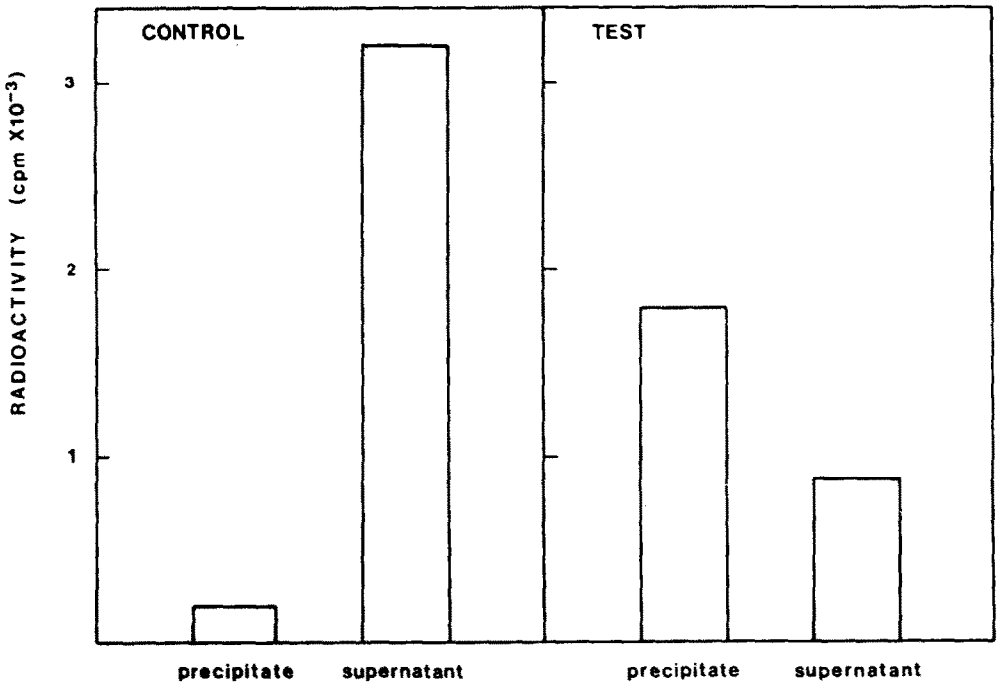

Fig. 5. Immuno precipitation of poly (dT)-transferrin/[^{3}H] poly (dA)-tailed pBR322 DNA hybridisation complex. [^{3}H] Poly (dA)-tailed pBR322 DNA (7 fmole, 12000 cpm) was incubated with 70 fmole [^{3}H] poly (dT)-transferrin* (400 cpm) (TEST) in the presence of carrier transferrin (10 μg per incubation mixture) in a final volume of 70 μl PBS. After 2 hr at room temperature anti transferrin antibody (150 μg) was added to each mixture. After 4 hr at 8° mixtures were centrifuged (9000 g/5 min) and precipitates and supernatant counted for radioactivity in scintillation fluid. * Concentration with respect to transferrin.

nucleoprotein complex at 37° which may arise from interactions between the duplex DNA and transferrin subsequent to the hybridisation event. The lability of the phosphoramide bond in the complex is unknown.

DISCUSSION

In a recent communication [2] we reported on the preparation of nucleoprotein complexes from the carbodiimide treated protein ligand transferrin and plasmid DNA intended for model studies on ligand directed DNA transfer into eukaryotic cells. We have described here the formation of non-covalent protein–DNA complexes resulting from the hybridisation of complimentary homopolymeric deoxyoligonucleotide tracts located on the participating protein and DNA species.

In this approach, the protein ligand transferrin has been modified by the 5′-attachment of oligo(dT) fragments. The required oligothymidylates were synthesized by a carbodiimide mediated polymerisation of 5′-TMP in the presence of 3′-*O*-acetyl-5′-TMP to minimise intramolecular reactions which lead to unwanted cyclic oligomeric structures [14]. The separation of homologues was conveniently and rapidly achieved by HPLC ion pair chromatography using the volatile buffer TEAB [15]. Fragment lengths were established by partial snake venom digestion [16] followed by separation of products by HPLC.

The direct coupling of oligo(dT) to transferrin by a water soluble carbodiimide was not attempted because of the tendency these agents have to derivatise carboxyl functions on the proteins to *N*-acylureas [3] which could lead to DNA binding by an electrostatic mechanism [2]. It has been reported by Orgel and coworkers that polynucleotides which have been converted to their phosphorimidazolide derivatives react readily with primary amines to form stable phosphoramidates [17]. We have used this approach to prepare $(pT)_5$-5′-transferrin through the intermediate imidazolide of $(pT)_5$ which undergoes a displacement reaction with transferrin. The 5′-anchorage of $(pT)_5$ offers a more favourable orientation of the oligomer for annealing to poly(dA)-tailed linearised pBR322 DNA than 3′-anchorage. It has been reported, however, that oligothymidylates shorter than the pentamer $(pT)_5$ fail to bind poly(A) at 0° [18] and for this reason no hybridisation studies were attempted using $(pT)_5$-5′-transferrin. Rather, the 3′ termini of $(pT)_5$-5′-transferrin were extended homopolymerically using terminal transferase to afford $(pT)_{300}$-5′-transferrin. The chain extension reaction conducted at 37° was terminated after 30 min as significant loss of growing poly(dT) chains through hydrolysis of the phosphoramide bond was noted on continued incubation. Poly(dT)-transferrin was however quite stable at 25° (<5% hydrolysis after 4 hr).

Poly(dT) transferrin successfully annealed with poly(dA)-tailed pBR322 DNA to afford complexes which, in contrast with poly(dT)-transferrin, were stable at 37°. This may be the result of conformational accommodation after hybridisation

which would diminish the importance of phosphoramide bond integrity.

In a preliminary binding study not described here specific recognition of poly(dT)-transferrin-[^{3}H] poly (dA)-tailed pBR322 DNA by transferrin receptors on rabbit bone marrow cells has been demonstrated. More detailed cell binding studies are in progress.

REFERENCES

1. S. Y. Cheng, G. T. Merlino and I. Pastan, *Nucleic Acids Res.* **11**, 659 (1983).
2. B. Huckett, H. Gordhan, R. Hawtrey, N. Moodley, M. Ariatti and A. Hawtrey, *Biochem. Pharmac.* **35**, 1249 (1986).
3. R. Timkovich, *Analyt. Biochem.* **79**, 135 (1977).
4. R. Hawtrey, M. Ariatti and A. O. Hawtrey, *IRCS Med. Sci.* **14**, 380 (1986).
5. M. Ariatti, R. Hawtrey, Z. Khan and A. O. Hawtrey, *IRCS Med. Sci.* **14**, 382 (1986).
6. H. G. Khorana and J. P. Vizsolyi, *J. Am. chem. Soc.* **83**, 675 (1961).
7. R. H. Hall, in *The Modified Nucleosides in Nucleic Acids*, p. 202. Columbia University Press, New York (1971).
8. R. Roychoudhury, E. Jay and R. Wu, *Nucleic Acids Res.* **3**, 863 (1976).
9. T. Maniatis, E. F. Fritsch and J. Sambrook, in *Molecular Cloning*, p. 105. Cold Spring Harbour Laboratory (1982).
10. C. R. Merril, D. Goldman, S. A. Sedman and M. H. Ebert, *Science* **211**, 1437 (1981).
11. D. C. Hinkle and M. J. Chamberlin, *J. molec. Biol.* **70**, 157 (1972).
12. G. Zubay, in Methods in *Enzymology*, Vol. 65 (Eds. L. Grossman and K. Moldave), p. 856. Academic Press, New York (1980).
13. R. M. Myers and R. Tjian, *Proc. natn. Acad. Sci. U.S.A.* **77**, 6491 (1980).
14. H. G. Khorana, J. P. Vizsolyi and R. K. Ralph, *J. Am. chem. Soc.* **84**, 414 (1962).
15. J. Porath, *Nature, Lond.* **175**, 478 (1955).
16. F. Sanger, J. E. Donelson, A. R. Coulson, H. Kossel and D. Fischer, *Proc. natn. Acad. Sci. U.S.A.* **70**, 1209 (1973).
17. B. C. F. Chu, G. M. Wahl and L. E. Orgel, *Nucleic Acids Res.* **11**, 6513 (1983).
18. R. Naylor, P. T. Gilham, *Biochemistry* **5**, 2722 (1966).