A Method for Linking Fluorescent Labels to Polynucleotides: Application to Studies of Ribosome–Ribonucleic Acid Interactions†

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ABSTRACT: A general procedure is described for introducing fluorescent labels into polynucleotides. The method utilizes the bisulfite-catalyzed transamination reaction of cytosine. Starting with poly(ribocytidylic acid), we have prepared polynucleotides containing various proportions of uracil, cytosine, and cytosine attached to a fluorescent label (nitrobenzofurazan). These fluorescent polynucleotides bind both 30S and 70S ribosomes from *Escherichia coli*; a large fluorescence enhancement is observed (50–100%). Competition experiments demonstrate that the fluorescent label weakens the polynucleotide binding by less than a factor of 2. Further, ribosomal 30S subunits which have been depleted of protein S1 (a protein probably involved in messenger RNA binding) bind the fluorescent polymers but do not alter the label fluorescence. Purified S1 itself binds the modified polynucleotides with a similar fluorescence enhancement as that of the 30S subunits. Therefore, S1 is probably the only ribosomal component that interacts with the fluorescent label. Applications of the labeling procedure to studies of synthetic and natural messenger RNA binding to ribosomes are discussed. A survey of the optical properties of the labeled polynucleotides shows that the label fluorescence at some wavelengths is very sensitive to protonation and base-pairing interactions of the cytosine base. A single strand specific polynucleotide binding protein (the gene 32 product of bacteriophage T4) also induces significant fluorescence changes in the attached label. These properties suggest applications of this labeling procedure to studies of polynucleotide conformations and polynucleotide–protein interactions.

The steps a ribosome uses to find the initiation site on a messenger RNA and begin translation constitute an important recognition reaction; translational efficiency contributes to the overall level of gene expression in a cell. Much remains to be learned about this process. How different features of the mRNA contribute to the association rate and initiation complex stability and how the rate is influenced by initiation factors and ribosomal components are largely unanswered questions. Unfortunately, quantitative studies of translational initiation are hampered by the problem of detecting ribosome binding to the initiation site in a way that allows equilibrium and kinetic measurements to be made in the necessary ranges (binding constants $>10^7$ M^{-1} and time scales of seconds).

In principle, a way to detect ribosome binding is to introduce a fluorescent "reporter group" into the mRNA in such a way that it undergoes a significant change in fluorescence intensity upon interaction of the mRNA with ribosomes; the binding reaction could then be followed optically with considerable sensitivity. Although there has been considerable interest in developing fluorescent probes for use in biological studies of nucleic acids [see, for example, reviews by Canter & Tao (1971) and Leonard & Tolman (1975)], few methods for fluorescent modifications of polynucleotides at internal positions have been reported. In one study a fluorescent alkylating reagent was reacted with poly(A), and the binding of ribosomes was examined (Pochon & Ekert, 1973); the fluorescence changes detected were too modest to be of extensive use. Another modified base, ethenoadenine, is of potential use in polynucleotide binding protein studies, but its fluorescence intensity is low when incorporated into polynucleotides (Tolman et al., 1974).

A promising method for modifying polynucleotides was reported some time ago by Shapiro & Weisgras (1970), who demonstrated the utility of bisulfite in catalyzing transamination at the N_4 position of cytosine with a variety of reagents. In this paper we describe the use of this reaction to attach a fluorescent label (NBF)[1] to synthetic polynucleotides. Some properties of the resulting polymers make them attractive for studies of ribosome–mRNA interactions: ribosomes bind them accompanied by a large fluorescence enhancement, the presence of the fluorescent label has only a minor effect on binding, and the fluorescence change observed is attributable entirely to interaction of the label with a single ribosomal component (protein S1) already known to be involved in natural mRNA binding. Some other optical properties of the derivatives described here may make them useful in studies of other binding proteins and as probes of polynucleotide conformation.

Materials and Methods

Materials. Poly(C) was obtained from Calbiochem; NBF-Cl (4-chloro-7-nitrobenzofurazan, also named 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole) and diaminopropane were from Aldrich. Sodium metabisulfite and other salts were reagent grade.

Buffers. All solutions were made up in doubly distilled water and filtered through Millipore HAWP before use. The buffer used for ribosome and S1 titrations contained 10 mM Tris, pH 7.7, 5 mM $MgSO_4$, 100 mM NH_4Cl, and 3 mM 2-mercaptoethanol ("titration buffer").

Ribosomes and S1 Protein. High salt washed ribosomes from *Escherichia coli* B were prepared essentially by the procedure of Kurland (1966), and subunits were separated by band sucrose gradient sedimentation. The isolated 30S subunits were pelleted, resuspended in titration buffer containing

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[1] Abbreviations used: NBF-Cl, 4-chloro-7-nitrobenzofurazan; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; 30S-S1, 30S ribosomal subunits specifically depleted of protein S1.

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15 mM $MgSO_4$ and 300 mM NH_4Cl, and activated at 37 °C (Zamir et al., 1971) before storing in small aliquots at −70 °C. Subunits specifically depleted of protein S1 were prepared by dialysis against 1 mM Tris (pH 7.7), 50 μM $MgSO_4$, and 3 mM 2-mercaptoethanol (Tal et al., 1971). The dialyzed subunits were pelleted and resuspended in the same buffer used for activation.

The S1 content of the different subunits prepared was checked on $NaDodSO_4$–polyacrylamide gels. The S1 peaks were quantitated on a scanning densitometer and compared with bands of known quantities of purified S1 run on the same slab gel. For the 30S preparations used in the titrations shown here, the S1 contents were 80% (30 S) and 5% (30S-S1). Subunit concentrations were calculated from the absorbance at 260 nm, by using the extinction coefficient 1.31×10^7 OD/M (Hill et al., 1969).

S1 protein was purified by the DNA–cellulose procedure and an extinction coefficient of 4.08×10^4 OD/M at 280 nm used (Draper & von Hippel, 1978). The purified protein was better than 95% active in its ability to bind denatured DNA–cellulose and poly(C)–cellulose.

Fluorescence Measurements. All fluorescence measurements were made on a Perkin-Elmer MPF-43 spectrofluorometer, equipped with a circulating water bath thermostated at 25 °C. A 4 × 4 mm quartz cuvette was used; sample volumes for titrations were 200 μL. Spectra are not corrected for variations in xenon lamp output or photomultiplier response with wavelength. For titrations, excitation and emission wavelengths were set at 478 and 550 nm, respectively, and the sample fluorescence was read relative to a standard as described previously (Draper & von Hippel, 1978). Unless otherwise indicated, titrations were performed in titration buffer.

In order to calculate a binding isotherm for ligands titrating a long lattice polynucleotide with the signal (fluorescence change) indicating the degree of polynucleotide saturation, the ligand site size must be known. For titrations involving very large binding constants or very high lattice concentrations (stoichiometric binding conditions), a plot of fluorescence change as a function of added ligand gives the site size when the initial slope is extrapolated to the maximum fluorescence change. In most situations completely stoichiometric binding is not practical to attain, and the initial slope of the plot is less than the maximum obtainable, with a corresponding underestimation of the site size. However, if the titration is performed at two different polynucleotide concentrations, the true site size n is given by

$$n = n_1 \frac{m_1/m_2 - 1}{P_1/P_2 - m_1/m_2}$$

where m_1 and m_2 are the initial slopes obtained at polynucleotide concentrations P_1 and P_2 and n_1 is the apparent site size calculated for the titration using P_1.

The maximum fluorescence enhancement was estimated by plotting the last titration points as a double-reciprocal plot and extrapolating to infinite ligand concentration. For most titrations four or five linear points were obtained.

Binding constants (K) and cooperativity parameters (ω) were calculated from titrations by fitting the McGhee & von Hippel (1974) "overlap" equations to a Scatchard plot of the data, with n (site size) fixed at the value calculated as described above. The overlap equation takes into account the fact that the concentration of free polynucleotide binding sites is not a linear function of the ligand bound, since large ligands ($n > 1$) cover more than one potential binding site.

Synthesis of Labeled Polynucleotides. The transamination and deamination reactions are accomplished by dissolving poly(C) (1–3 mg/mL) in buffer adjusted to the desired pH and containing the appropriate concentrations of $NaHSO_3$ and diaminopropane (see Table I) and 0.1 M of either Mes or Mops buffer. Hydroquinone (5 mM) is included to take up free radicals formed by bisulfite (Hayatsu & Miller, 1972). The solution should be made up fresh for each use.

The solution is incubated at 42 °C for the desired time, and the reaction is quenched by adding sufficient NaOH to bring the pH to 9.0. After 1 h at room temperature, the mixture is desalted over Sephadex G-75 equilibrated with 0.1 M ammonium acetate. The extent of bisulfite removal (generally complete at this point) can be checked by examining the polynucleotide UV spectrum (Shapiro & Braverman, 1972). To ensure complete bisulfite removal, we dialyzed the polynucleotide fractions against 100 volumes of 1 mM sodium borate.

At this point the extent of transamination and deamination can be roughly assessed by hydrolyzing a sample of the polymer in 1 M HCl at 42 °C for 2 h, spotting the sample on a cellulose thin-layer plate, and developing the chromatogram in 65% 2-propanol–2 N HCl. Approximate R_f values for nucleotides in this system are 0.8 (UMP), 0.5 (CMP), and 0.3 [N^4-(3-aminopropyl)cytidine monophosphate].

Reaction of NBF-Cl with the primary alkyl amines now present on the polynucleotide does not proceed satisfactorily in aqueous solution: at a pH high enough to give a significant concentration of unprotonated amine, NBF-Cl is rapidly degraded by base and significant hydrolysis of the polymer occurs before labeling is complete. To circumvent this problem, we obtained the deprotonated form of the polynucleotide in ethanol, as the cetyltrimethylammonium salt (Weil & Ebel, 1962). To a solution of the polynucleotide on ice are added in rapid succession NaOH to 0.1 M and a threefold molar excess of cetyltrimethylammonium bromide over the RNA phosphates. After we spin down the precipitate and drain and dry the pellet carefully, the polynucleotide dissolves easily in absolute ethanol. Precipitation from high pH is required for a complete reaction of the amines in the next step.

For the reaction with NBF-Cl, a polynucleotide concentration of about 5–10 A_{260} units/mL is optimum. A 15-fold excess of triethylamine over RNA phosphates is added, followed by NBF-Cl to 20 mg/mL (the NBF-Cl can first be dissolved to high concentration in a small amount of Me_2SO). The solution is incubated at 30 °C in the dark (NBF-Cl is photosensitive in base), adding an additional 15-fold excess of triethylamine every hour to take up protons generated by the NBF reaction. At the end of 3 h, the polynucleotide is precipitated by adding 5 μL of 4 M NaCl per mL of solution. After being allowed to sit a few minutes, the precipitate is spun out and the supernatant is discarded. The pellet dissolves readily in water buffered to pH 7.2 with Mops. A large amount of unreacted label is usually present at this point. It can be removed either by extracting the solution several times with chloroform or by precipitating the polymer again with cetyltrimethylammonium bromide, redissolving in ethanol, and dripping this solution into several volumes of molar ammonium acetate. Finally, the polynucleotide is dialyzed against 10 mM ammonium acetate, lyophilized if desired, and stored at −20 °C.

As a small amount of degradation occurs during these procedures, the size of the polymers is checked by band sedimentation on sucrose gradients, as described by Burgi & Hershey (1963).

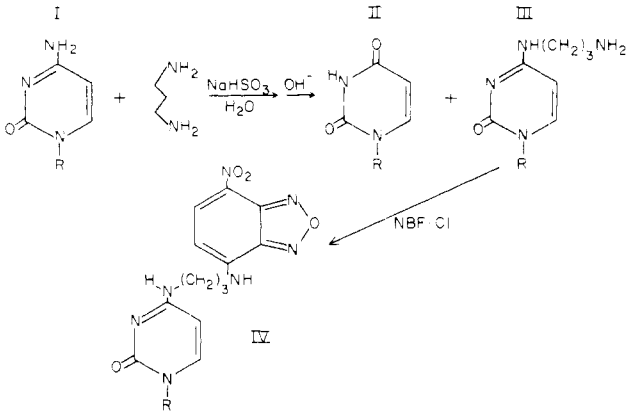

FIGURE 1: Scheme for introducing a fluorescent label into poly(cytidylic acid). See the text for an explanation.

A control reaction with unmodified poly(C), precipitated from base and reacted with NBF-Cl as described above, showed no detectable incorporation of NBF. In another control, the possibility that diaminopropane can cross-link two cytidine residues was checked. Poly(C) was modified to >80% with diaminopropane, and the isolated product was hydrolyzed to mononucleotides. The hydrolysate was chromatographed on Bio-Gel P2. No peak was found which would correspond to two nucleotides cross-linked by diaminopropane. By use of this same procedure, 0.5% cross-linking was detected with CMP reacted at 35 mg/mL.

Monomer and Polymer Extinction Coefficients. The derivative N^4-(3-aminopropyl)cytidine 5′-monophosphate was prepared as described above, incubating 5′-CMP in buffer containing 3 M diaminopropane and 1 M $NaHSO_3$ at pH 6.2 for 30 h at 42 °C. The derivative was isolated by chromatography on Bio-Gel P_2, followed by repeated precipitation from ethanol. The reaction with NBF-Cl was carried out in ethanol–H_2O (2:1) with 0.1 M triethylamine. After the solution was neutralized, it was loaded onto a silica gel column and rinsed thoroughly with ethanol, and the product was eluted with distilled water. The product gave a single, fluorescent spot on a cellulose thin-layer plate developed in 65% 2-propanol–2 N HCl, with R_f = 0.8.

The extinction coefficient of NBF–CMP was determined by digesting with alkaline phosphatase and determining inorganic phosphate by a modification of the method of Brunette et al. (1978). Absorption maxima and extinction coefficients at pH 7.2 in 5 mM Mops buffer are as follows: 271 nm and 11 500 OD/M; 348 nm and 5710 OD/M; 483 nm and 17 500 OD/M.

Extinction coefficients and percent compositions for poly(U,NBF–C) or poly(C,NBF–C) were calculated by hydrolyzing a sample of polynucleotide in 1 M HCl for 2 h at 42 °C and using the following mononucleotide extinction coefficients (in acid solution): NBF–CMP, 260 nm and 7840 OD/M and 476 nm and 17 000 OD/M; CMP, 260 nm and 6500 OD/M; UMP, 260 nm and 10 000 OD/M.

Other polynucleotide extinction coefficients used were as follows: poly(U), 9500 OD/M at 260 nm; poly(C), 6000 OD/M at 268 nm; poly(A), 10 500 OD/M at 258 nm; poly(I), 10 200 OD/M at 248 nm.

Results and Discussion

Synthesis of Fluorescent-Labeled Polynucleotide. The general scheme for introducing a fluorescent label onto a cytosine residue is shown in Figure 1. Bisulfite adds across the 5,6 double bond of cytosine (I) and allows exchange of the exocyclic amino group with other amines in solution; after exchange the double bond can be restored by treatment with mild base. If diaminopropane is used, the net effect of the reaction is to introduce an aminopropyl moiety at the N_4 position of cytosine (III). The primary alkyl amine can then be reacted with any of a variety of fluorescent reagents (shown is NBF-Cl, IV). In the presence of bisulfite, cytosine also deaminates to produce uracil (II). By adjusting the reaction conditions, we can prepare polynucleotides with any proportion of cytosine, uracil, and N^4-(3-aminopropyl)cytosine.

Table I: Poly(C) Modifications by Bisulfite and Diaminopropane[a]

incubn time (h)	[diaminopropane]	pH	% UMP	% CMP	% AP-CMP[b]
24	1	5.5	89	0	11
144	1	5.5	93	0	6.6
31	1	5.8	77	0	23
72	1	7.2	0	90	9.9
30	3	6.2	0	0	100

[a] Incubation of poly(C) was at 42 °C in solutions containing 1 M $NaHSO_3$ and the indicated molar concentration of 1,3-diaminopropane at the indicated pH for the time shown. Further details, and the method for determining the polymer composition, are given under Materials and Methods. [b] N^4-(3-Aminopropyl)-cytidine monophosphate.

Table I shows the base composition of the polynucleotides obtained after reacting poly(C) with bisulfite and diaminopropane at various concentrations and pH (see Materials and Methods for details). Both deamination and transamination are slowed considerably as the pH is raised, but the rate of transamination relative to deamination rises. Thus, at pH 5.5, with molar bisulfite and diaminopropane, deamination is heavily favored and the cytosine is completely consumed in less than 24 h. The same concentration of reactants at pH 7.2 gives exclusively transamination, and the reaction has not gone to completion after several days. Increasing the diaminopropane concentration favors the transamination reaction, while higher bisulfite concentrations favor deamination. Some aspects of these reactions have been studied in detail (Shapiro et al., 1974; Shapiro & Weisgras, 1970).

Nitrobenzofurazan chloride was chosen for the fluorescent label for several reasons. It is reasonably soluble in water; its absorption after reaction with amines is intense and is in the visible region, well removed from absorption by protein or nucleic acids; its fluorescence intensity is very sensitive to the environment; compared to other reagents available, it is small. At the basic pH necessary for reacting NBF-Cl with amines, hydrolysis of the reagent competes with amination; this problem was circumvented by carrying out the reaction in ethanol with the cetyltrimethylammonium salt of the polynucleotide (details are given under Materials and Methods).

Optical Properties of the Fluorescent Derivatives. Figure 2 shows the absorption spectrum of the mononucleotide derivative NBF-CMP. Three maxima are observed. The shortest wavelength peak (271 nm) is mostly due to the cytosine absorption, with some contribution from the label (20%); the two longer wavelength peaks (348 and 482 nm) derive entirely from the NBF label. The fluorescence spectrum also shows three peaks (Figure 3), corresponding to the three absorption peaks. One emission maximum at 551 nm is seen at all excitation wavelengths. The shortest wavelength excitation peak (I) was unexpected since derivatives of NBF with alkyl amines or amino acids (not shown) show almost no fluorescence intensity in this region of the spectrum. It is likely that this excitation peak arises from energy transfer between the cytosine base and the label, as discussed below.

Table II: Summary of Fluorescence Spectra of NBF–Cytosine Derivatives[a]

derivative and conditions	excitation peaks[b] I λ_{max}	I I^i	II λ_{max}	II I^i	III λ_{max}	III I^i	emission peak[c] λ_{max}	emission peak[c] I^i
NBF-CMP, pH 7.2[d]	279	1.00	349	1.00	476	1.00	551	1.00
NBF-CMP, pH 2.0	290	1.63	345	1.11	470	1.33	547	1.33
NBF-CMP, pH 7.2, 50% ethanol	278	2.45	345	2.78	469	3.21	542	3.22
poly(C,NBF-C), pH 6.8[e]	277	1.00	352	1.00	477	1.00	548	1.00
poly(C,NBF-C), pH 5.0	281	3.15	350	1.39	476	1.12	547	1.12
poly(C,NBF-C), pH 2.1	287	2.30	346	0.81	470	0.81	546	0.78
poly(C,NBF-C) + poly(I)[f]	277	2.19	354	1.59	483	1.36	546	1.37
poly(U,NBF-C)[g]	277	1.00	350	1.00	473	1.00	546	1.00
poly(U,NBF-C) + gp32	280	1.31	350	0.87	473	0.76	546	0.77
poly(U,NBF-C) + 30 S	280	2.46	354	2.30	485	1.84	547	1.83
poly(U,NBF-C) + S1	281	3.12	356	3.05	485	2.61	547	2.70
poly(U,NBF-C) + RNase[h]	279	0.53	350	0.73	475	0.98	550	0.98

[a] These spectral data are uncorrected for instrument characteristics; therefore, the wavelength maxima are not necessarily those of the absorption maxima. In each of the three sections of the table, the intensities of each peak are reported relative to the intensities in the first row. [b] Emission observed at 550 nm. [c] Excitation at 478 nm. [d] Buffer is 10 mM Mops; adjustment to pH 2.0 was made with HCl. [e] The polymer is 9.9% modified and is in 50 mM sodium acetate buffer. As the solution was titrated with HCl, the fluorescence intensity reached a maximum at pH 5.0 and declined as the pH was lowered further. [f] Buffer is 100 mM sodium acetate, pH 6.8. The fluorescence spectrum was taken after titrating to an I/C ratio of 1.23. [g] Polymer is 6.6% modified and is in titration buffer. gp32 is the phage T4 gene 32 product and was added to a saturating concentration. Titration with 30S subunits and S1 protein was to ~60% saturation; the intensities reported are extrapolated to complete saturation. [h] Pancreatic ribonuclease was added to 30 μg/mL, and the spectrum was taken 15 min after addition. [i] I, intensity.

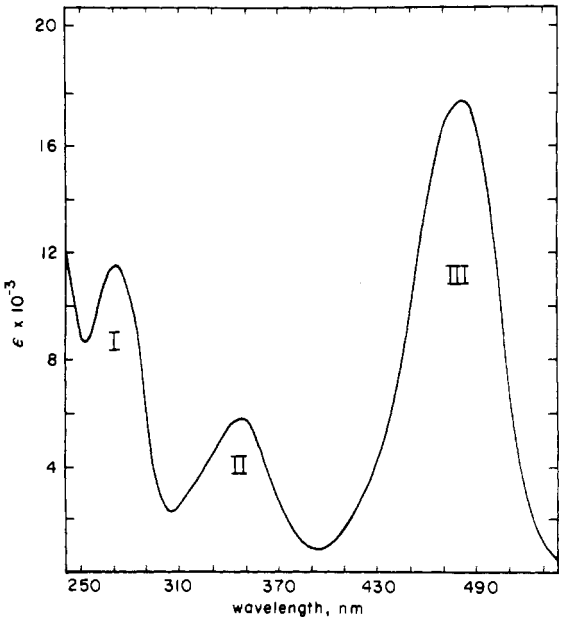

FIGURE 2: Absorption spectrum of NBF–CMP, in 5 mM Mops buffer, pH 7.2.

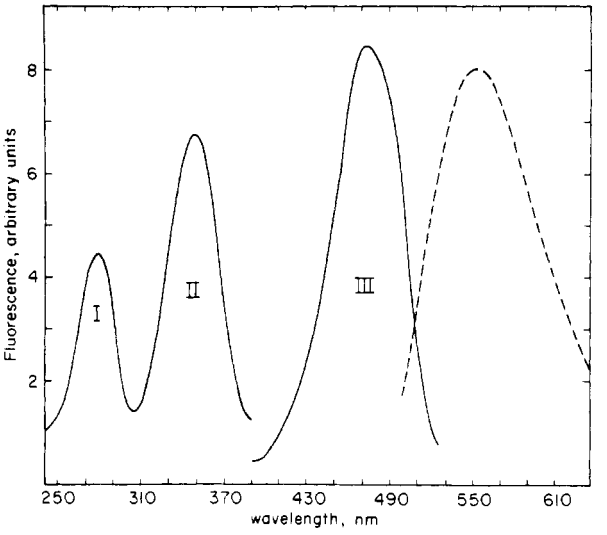

FIGURE 3: Excitation and emission spectra of NBF–CMP. For the excitation spectrum, the emission wavelength was 550 nm (—); the excitation wavelength was 478 nm for the emission spectrum (---). The mononucleotide concentration was 1.3 μM in 5 mM Mops buffer, pH 7.2. The excitation spectrum is uncorrected for the lamp spectrum; hence, the excitation maxima do not correspond exactly to the absorption maxima shown in Figure 2. At wavelengths greater than 390 nm, the instrument sensitivity is decreased by a factor of 3.

Factors Affecting NBF Fluorescence. The relative fluorescence intensities and (uncorrected) wavelength maxima of several NBF derivatives under different conditions are tabulated in Table II. These data demonstrate different ways in which the NBF fluorescence is altered by its environment and by interactions of the nucleotide base to which it is linked.

The fluorescence intensity of NBF is known to increase substantially in organic solvents (Ghosh & Whitehouse, 1968). This is demonstrated here by comparing the fluorescence of NBF–CMP in aqueous buffer and 50% ethanol. A substantial fluorescence enhancement (about threefold) is seen across the entire excitation spectrum, together with a small blue shift in the excitation and emission maxima.

The excitation peak I seems to be particularly sensitive to protonation and base-pairing interactions of the cytidine base. CMP is protonated at the N_3 position with a pK of 4.5. Titrating NBF–CMP with acid gives a substantial fluorescence enhancement of peak I with an apparent pK of 4.6. The peak wavelength is also shifted by 11 nm to the red, the same shift as occurs in CMP (and NBF–CMP) absorbance upon protonation. This shift suggests that energy absorbed by the cytosine base is able to excite the NBF label.

Restricted rotation about the exocyclic nitrogen–ring carbon bond of cytosine should allow two isomers of the NBF–cytosine derivatives to exist: one presenting a hydrogen in the Watson–Crick base-pairing position [the anti (with respect to N_3) isomer, drawn in Figure 1] and the other having the alkyl chain in a position that interferes with base pairing (syn isomer). High-resolution nuclear magnetic resonance measurements on N^4-methylcytosine have demonstrated that the syn isomer is the preferred orientation by 20:1 and that this weakens the cytosine–guanine base pair by 1.0 kcal (Engel & von Hippel,

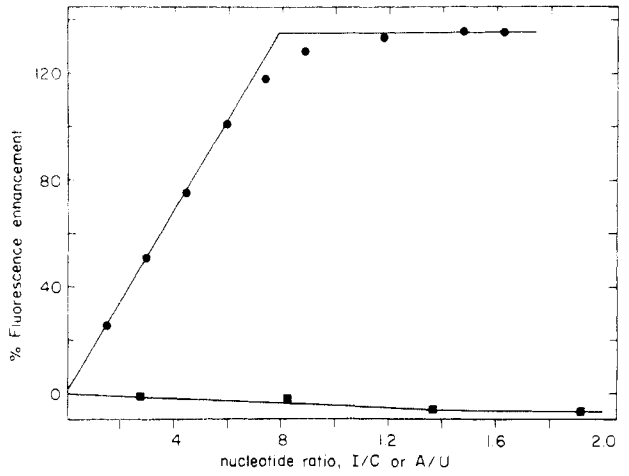

FIGURE 4: Titration of poly(C,NBF–C) with poly(I). The titration was performed in 200 mM NaCl and 25 mM Mops, pH 7.2, at 20 °C. The poly(C,NBF–C) concentration was 8.20 μM in nucleotides. Fluorescence excitation was at 277 nm, with the emission read at 550 nm.

1974). Thus, partial substitution of N^4-methylcytosine into poly(C) lowers the melting temperature of a poly(C)–poly(I) complex by 0.5 °C for every percent substitution [Brimacombe & Reese (1966) and see discussion in Engel & von Hippel (1974)]. Though the NBF moiety is a relatively bulky substituent, the propyl chain attaching it to the cytosine amine is long enough to allow it to lie in the major groove of a Watson–Crick helix without sterically interfering with base pairing. It therefore seems likely that poly(C,NBF–C) derivatives will be able to form normal base-paired structures, though probably with lower stability than poly(C).

The fluorescence changes accompanying two different base-pairing interactions of poly(C,NBF–C) are shown in the second section of Table II. At acid pH poly(C) forms an antiparallel, double-helical structure containing one proton shared between the N_3 positions of every base pair; each base pair forms hydrogen bonds at three positions, including N_4. The transition to a double helix occurs with a pK of 5.7, accompanied by protonation of half the bases, and the helix is disrupted at lower pH (pK = 3.0) upon protonation of all the bases (Hartman & Rich, 1965). Similar behavior is seen with poly(C,NBF–C), as indicated by changes in the NBF fluorescence. Peak I again shows the largest changes, reaching a maximum at pH 5.0 and declining at lower pH to an intensity expected for protonated NBF–cytosine. The peak I maximum shifts to the red during the titration, as expected from the acid titration of NBF–CMP; the shift is nearly half complete at pH 5.0 (Table II). These data suggest that poly(C,NBF–C) forms the same helical, half-protonated structure in acid as poly(C), though with weaker stability as indicated by the narrower pH range over which the structure is formed.

The sensitivity of peak I to base pairing is also demonstrated by titrating poly(C,NBF–C) with poly(I), as shown in Table II and Figure 4. [Poly(I) forms two hydrogen bonds with poly(C), at the cytosine N_3 and N_4 positions.] Peak I fluorescence increases linearly with added poly(I) until a plateau is reached at a nearly equimolar ratio of the two polymers. To show what happens to the NBF fluorescence in the case of a mismatch, we titrated poly(U,NBF–C) with poly(A); only a slight quenching of fluorescence is seen. A control titration (not shown), performed identically except for the presence of 13 μM ethidium bromide, demonstrated by the increase in ethidium fluorescence (excited at 525 nm) that A·U base pairs were formed under the conditions used (Morgan et al., 1979). This sensitivity of the NBF label to the interactions of the base it is attached to could make it useful for a probe of RNA structure or (for example) for observing tRNA anticodon–codon interactions. Further studies are in progress to determine the amount by which the NBF label affects base pairing.

The NBF fluorescence is also affected by the binding of protein ligands, as shown in the third section of Table II. The phage T4 gene 32 product binds to the backbone of single-stranded DNA and RNA, holding the bases in an unstacked conformation but probably not interacting with the bases themselves (Jensen et al., 1976). Addition of this protein to poly(U,NBF–C) quenches the NBF fluorescence at most wavelengths, with no shift in the excitation or emission maxima. (The peak appearing at 280 nm probably derives from energy transfer from gene 32 protein tryptophans rather than an enhancement of the 277-nm peak.) The label fluorescence must be somewhat sensitive to the presence of adjacent nucleotides in a polymer, since digesting poly(U,NBF–C) with ribonuclease lowers the fluorescence. Possibly the gene 32 protein holds the polynucleotide in such a conformation that the label fluorescence is quenched slightly, without actually interacting with the label.

Interactions with Ribosomes. 30S ribosomal subunits have a much more dramatic effect on poly(U,NBF–C) fluorescence: a substantial increase in intensity, together with a shift to longer wavelengths, is seen for all the excitation peaks. The magnitude of this enhancement suggests that the ribosome interacts directly with the label. At some point along the length of the ribosomal mRNA binding site, the bases of the mRNA (rather than merely the backbone) must be bound to the surface or interior of the particle to interact with tRNA. It is therefore not surprising that some of the labels are placed in an environment on the ribosome very different from the surrounding buffer. The specificity of this interaction in relation to the ribosomal protein S1 will be discussed below.

To follow the binding of ribosomes to the fluorescent polynucleotides, we have used an excitation wavelength of 478 nm. Even though the percentage change in fluorescence intensity is smallest at this wavelength, the initial intensity is greater than at the other excitation maxima, and there is no interference by ribosome absorption of the excitation beam [the "inner filter" effect, Cantor & Tao (1971)]. Titrations of poly(U,NBF–C) and poly(C,NBF–C) are shown in Figure 5. In both cases, a pair of titrations at different polynucleotide concentrations extrapolate (at infinite ribosome concentration) to the same maximum fluorescence intensity and are fit by the same set of binding parameters [binding constant, site size, and cooperativity, as defined in McGhee & von Hippel (1974)].[2] For the titrations with poly(U,NBF–C), a small amount of cooperativity between the subunits must be assumed

[2] Both the method used for calculating the site size and the overlap equation used to determine K and ω (see Materials and Methods) assume an infinitely long polynucleotide lattice. This assumption can lead to serious miscalculations if the number of ligands able to bind a lattice is less than five to ten (Epstein, 1978). The two modified polynucleotides titrated in Figure 3 are both 250 nucleotides in length (5.0 S), and thus most of the polymers in a solution will be able to accommodate about five 30S subunits. Under these conditions the infinite lattice approximation will involve a 10–20% overestimation of the site size and perhaps a similar underestimation of the binding constant. A second factor affecting the accuracy of these results is the presence of 30S subunits with weak affinity for the polynucleotide (see the discussion of S1 protein), which will lead to an underestimation of both the site size and the binding constant. The magnitude of the error with this preparation of ribosomes is less than 20%.

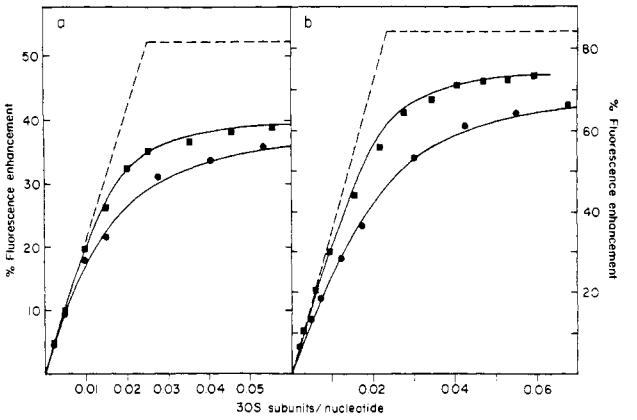

FIGURE 5: Titration of fluorescent polynucleotides with 30S ribosomal subunits. (a) Titration of poly(C,NBF–C) (9.9% modified) at 1.64 (●) and 8.20 μM (■). The solid lines are noncooperative, overlap binding isotherms calculated with $K = 6.0 \times 10^6$ M^{-1} and $n = 40$ bases. The dashed line shows the extrapolation used to calculate the site size, i.e., the binding curve predicted for an infinite concentration of polynucleotide. (b) Titration of poly(U,NBF–C) (6.6% modified) at 1.5 (●) and 6.32 μM (■). The data are fit with cooperative, overlap binding isotherms calculated with $K = 2.5 \times 10^6$ M^{-1}, $n = 42$ bases, and $\omega = 20$.

in order to fit the data (a cooperativity factor, ω, of 20). This may be only apparent and due to the relative shortness of the polynucleotide lattice, which gives a sharper titration (the lattice is easier to saturate) than expected for a (noncooperative) ligand of this site size binding a very long lattice [Epstein (1978) and see footnote 2]. However, no cooperativity is required to account for the titration of poly(C,NBF–C). It is also worth noting that others have detected apparent cooperativity with ribosomes binding poly(U) (by a filter binding assay); the cooperativity disappears if the poly(U) is short enough to bind only one subunit (Kirillov et al., 1978). This point is being investigated further by using a series of oligomers of increasing length.

The 70S ribosomal subunits titrate the fluorescent polymers with identical fluorescence enhancements as 30S subunits and with roughly the same binding parameters (data not shown).

To determine the degree to which the presence of the NBF label perturbs the polynucleotide binding, we performed competition experiments. First, addition of unlabeled polynucleotide to a complex of 30S subunits and fluorescent polynucleotide demonstrates that unmodified polymers compete for all of the fluorescence enhancement. An example is shown in Figure 6. All homopolymers that have been tried give complete competition [poly(U) and poly(C) against poly(U,NBF–C), and poly(U), poly(C), and poly(A) against poly(C,NBF–C)], though poly(U) is a much stronger competitor than poly(A) or poly(C). Therefore, the label is not directing the polynucleotide to an unusual binding site on the ribosome, but binding is to a site normally utilized by homopolymers.

In a second set of competition experiments, mixtures of labeled and unlabeled polynucleotides were titrated with 30S subunits; titrations of poly(U) mixed with poly(U,NBF–C) and poly(C) mixed with poly(C,NBF–C) are shown in Figure 7. The dashed curves drawn in the figure illustrate two possible outcomes. In the extreme case that the unlabeled competitor is a much more effective binder (and therefore is titrated before the labeled polymer), a lag will be observed in the titration curve. If the subunits have identical affinities for the two polynucleotides, the upper curve is predicted. The two titrations of Figure 7 clearly come closest to the equivalent binding case; at low saturation there is no indication of a lag

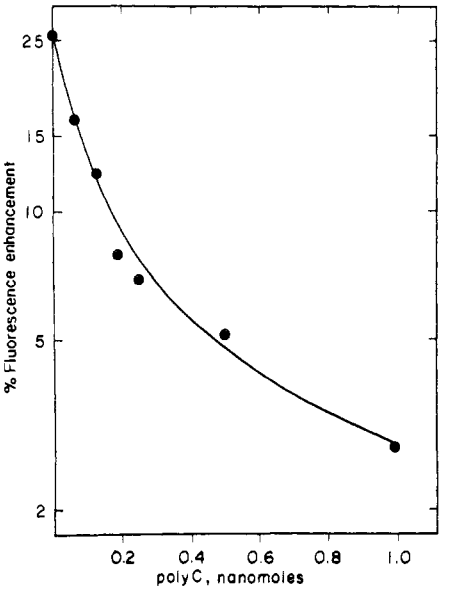

FIGURE 6: Competition titration. 1.6×10^{-10} mol of poly(C,NBF–C) (9.9% modified) in 0.2 mL was titrated first with 2.1×10^{-11} mol of 30S subunits, followed by aliquots of poly(C). The decline in the total fluorescence enhancement with successive additions of poly(C) is shown.

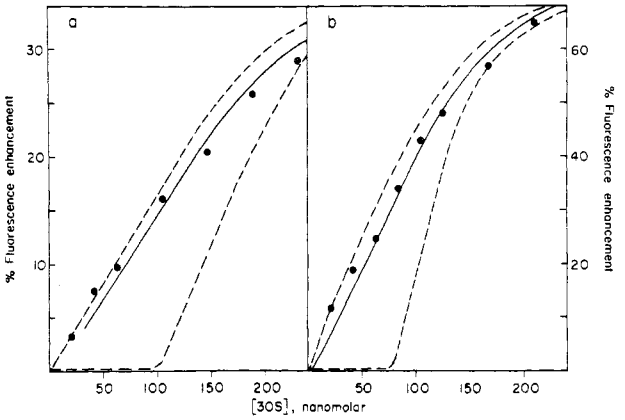

FIGURE 7: Titrations of mixtures of modified and unmodified polynucleotides. For both titrations the dashed lines illustrate the curves expected if the modified RNA binds much more weakly than the unmodified RNA (lower curve) or if the two bind with equivalent affinity (upper curve). The solid line is the curve predicted if the unmodified polymer binds twice as strongly as the modified polymer. (a) Titration of 8.20 μM poly(C,NBF–C) (9.9% modified) plus 3.59 μM poly(C). (b) Titration of 3.16 μM poly(U,NBF–C) (6.6% modified) plus 3.08 μM poly(U).

in the binding curve and the data deviate from the predicted curve only at higher saturation. The data at lowest saturation are the most accurate; they are less affected by variations in the maximum enhancement observed and by differences in the lengths of the labeled and unlabeled polynucleotides.[3] To show

[3] A large ligand saturates a short lattice more easily than a long one (Epstein, 1978); in a competition experiment between two different length polymers, this effect would show up as an increasing difference in affinity with increasing saturation. This effect could account at least in part for the deviation of the poly(C) data from the predicted curve at high saturation; the competitor in this case is about 7 S, compared to 5 S for the modified polymer. Cooperatively interacting ligands are more dependent on lattice length; the larger number of cooperative interactions possible on a long lattice gives a greater average binding constant per bound ligand than on a short lattice. This may account for the greater sensitivity of the poly(U) competitions to competitor length. In the competition shown in Figure 6, both labeled and unlabeled polymers are 5.0 S; longer poly(U) (6.7 S) is a significantly better competitor (by about twofold).

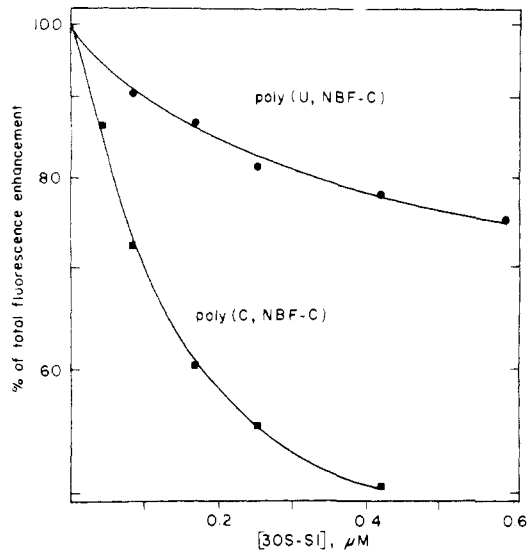

FIGURE 8: Competition between 30S and 30S-S1 subunits. Complexes between 0.15 μM 30S subunits (80% containing a copy of S1 protein) and a fluorescent polynucleotide were titrated with 30S-S1 subunits (5% containing S1 protein); the decline in the fluorescence enhancement is shown. (●) 3.16 μM poly(U,NBF–C), 6.6% modified; (■) 8.20 μM poly(C,NBF–C), 9.9% modified.

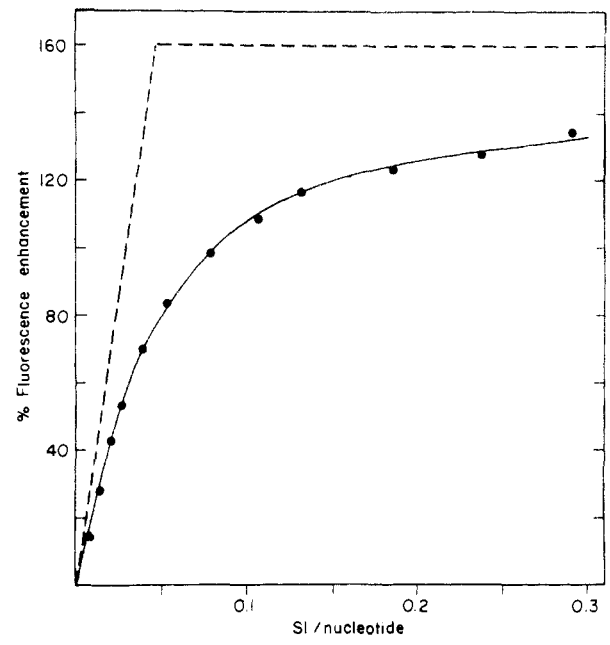

FIGURE 9: Titration of 1.26 μM poly(U,NBF–C) (6.6% modified) with purified S1 protein. The dashed line shows the titration predicted at infinite polymer concentration. The solid line is an overlap binding isotherm fit to the data with $K = 1.8 \times 10^6$ M^{-1}, $n = 21$ bases, and $\omega = 6.0$.

the sensitivity of the competition method to small differences in binding affinity, we have calculated the middle line drawn in the two titrations assuming that the unlabeled competitors bind subunits twice as strongly as do the labeled polymers. For the most part the data fall above this line. We conclude that the NBF–propyl moiety, at the level of modification used in these titrations, has at most a very minor influence on ribosome–RNA interaction. It should be noted that a difference of a factor of 2 in binding affinity corresponds to a difference in binding free energy of only 0.4 kcal, compared to the 9–10 kcal total free energy observed for the binding reaction.

Fluorescence Enhancement Depends on S1 Protein. The ribosomal protein S1 is probably involved in mRNA binding. In one of the first experiments to suggest this role for S1, van Duin & Kurland (1970) fractionated 30S subunits according to their ability to bind poly(U) tightly and found the tight binding fraction to be enriched in S1 protein. Since then cross-linking and chemical protection experiments have also suggested that S1 binds mRNA to the ribosome (Noller et al., 1971; Fiser et al., 1977), and a dramatic stimulation by S1 of poly(U) and MS2 RNA directed protein synthesis has been demonstrated (van Dieijen et al., 1975). Since 30S subunits, as normally isolated, contain a fractional copy number of S1, it is possible that the S1-deficient class is inactive in RNA binding. Therefore, we have tested 30S subunits specifically depleted of S1 by a low-salt treatment (30S-S1) for binding to the fluorescent polynucleotides.

In titrations of either poly(U,NBF–C) or poly(C,NBF–C) with 30S-S1 subunits, we have not detected any significant changes in the label fluorescence at any excitation wavelength (data not shown), although competition experiments demonstrate that the 30S-S1 subunits are indeed binding the polynucleotides. Figure 8 shows the titration of 30S–fluorescent polynucleotide complexes with 30S-S1 subunits. S1-deficient subunits compete for binding, but at different levels depending on the polymer titrated. 30S-S1 subunits bind only about twofold more weakly than 30S subunits when poly(C,NBF–C) is used, while the difference in affinity is at least a factor of 10 with poly(U,NBF–C).

The absence of any fluorescence changes with 30S-S1 subunits binding the fluorescent polynucleotides suggests that S1 provides the environment responsible for altering the NBF fluorescence. As further support for this idea, Figure 9 shows that purified S1 titrates poly(U,NBF–C) with a substantial fluorescence enhancement. The enhancement occurs at all wavelengths, with a small red shift in the excitation maxima (Table II), qualitatively similar to the fluorescence changes seen with 30S subunits. However, S1 binds poly(U,NBF–C) with a site size about half that of 30S subunits (21 bases, in contrast to ~40 bases). The closer packing of S1 should allow about twice as many fluorescent labels to interact with (purified) S1 as with S1 that is part of the 30S subunit, and in fact about twice as much enhancement is seen with S1 (161% instead of 84%). Hence, the influence of S1 on the NBF fluorescence is sufficient to account for the entire fluorescence enhancement seen with 30S subunits. Similar results are found if S1 is used to titrate poly(C,NBF–C): the site size is smaller (25 nucleotides) and the fluorescence enhancement is larger (122%) than those found for 30S subunits.

Conclusion

The properties of the NBF-labeled polynucleotides should make them useful for studies of ribosome interaction with synthetic polynucleotides. Ribosome binding can be followed at low concentrations (10^{-8} M in ribosome binding sites), and the label does not significantly perturb the polynucleotide binding. The titrations presented here already suggest some interesting characteristics of ribosome–homopolymer interaction, which relate to the role of S1 in protein synthesis and bear further investigation. Poly(U) binds 30S subunits very tightly (perhaps cooperatively) and in an S1-dependent manner, while poly(C) and poly(A) bind much more weakly and [for poly(C) at least] in a way much less affected by S1 protein. These properties parallel the ability of ribosomes to translate homopolymers: poly(U) is translated at a high level dependent on S1 protein, while poly(A) is translated poorly and is hardly affected by S1 (van Dieijen et al., 1975).

These studies on synthetic polymers have been conducted with the eventual goal of applying the labeling procedure to

natural mRNAs. The potential utility of the bisulfite reaction in this regard is its high specificity for single-stranded regions of an RNA molecule (Shapiro et al., 1973); this specificity of the transamination reaction has been used to advantage in studies of tRNA structure (Schulman et al., 1974). We imagine a method for limiting the modification reaction to one or a few bases in a large RNA: if a large RNA molecule is hybridized to a complementary DNA containing a small deletion, the bisulfite reaction will take place only at the looped out region of RNA corresponding to the deletion. An attempt to label the rIIB mRNA from bacteriophage T4 at specific sites is underway in this laboratory (Shinedling, Draper, and Gold, unpublished experiments).

It is probable that the site on S1 protein responsible for the fluorescence enhancement is used by natural mRNAs as well as synthetic homopolymers. S1 is essential for initiation complex formation with MS2 RNA (Kolb et al., 1977), and van Dieijen et al. (1978) have further argued from their studies on initiation in the presence of anti-S1 that S1 is required for the ribosome to recognize and bind the messenger initiation site. S1 is thus likely to interact with mRNA (and an appropriately placed label) at an early stage in initiation complex formation and provide a highly specific fluorescence signal (registering interaction of S1 with the mRNA) for kinetic studies of initiation in the presence of various initiation factors and initiator tRNA.

Finally, the versatility of the general reaction scheme described should be noted. Different lengths of alkyl spacers and a variety of fluorescent reagents with different chemical and fluorescence properties can be used to prepare labeled polynucleotides with various fluorescence properties. These should be of use in studies of the structure and interactions of a variety of polynucleotides and polynucleotide binding proteins.

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