hinge and from the active site, implies a delocalization of ligand-induced conformational transitions. Studies are in progress to evaluate the extent of delocalization of conformational changes in PGK.

ACKNOWLEDGMENTS

We are indebted to Dr. R. A. Hitzeman (Genentech, South San Francisco) for the yeast strains and yeast expression vector, Dr. R. Paxton for access to his HPLC equipment, and Dr. T. Lee and K. Legesse for mass spectral analyses.

REFERENCES

Bacharach, A. D. F., Markland, F. S., Pellino, A., & Weber, B. H. (1977) *Biochem. Biophys. Res. Commun. 74*, 165-171.

Banks, R. D., Blake, C. C. F., Evans, P. R., Haser, R., Rice, D. W., Hardy, G. W., Merrett, M., & Phillips, A. W. (1979) *Nature (London) 279*, 773-777.

Blake, C. C. F., Rice, D. W., & Cohen, F. E. (1986) *Int. J. Pept. Protein Res. 27*, 443-448.

Brevet, A., Roustan, C., Desvages, G., Pradel, L.-A., & van Thoai, N. (1973) *Eur. J. Biochem. 39*, 141-147.

Bücher, T. (1947) *Biochim. Biophys. Acta 1*, 292-314.

Chen, C. Y., Opperman, H., & Hitzeman, R. A. (1984) *Nucleic Acids Res. 12*, 8951-8970.

Hitzeman, R. A., Hagie, F. E., Hayflick, J. S., Chen, C. Y., Seeburg, P. H., & Derynck, R. (1982) *Nucleic Acids Res. 10*, 7791-7808.

Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.

Larsson-Raźnikiewicz, M., & Jansson, J. R. (1973) *FEBS Lett. 29*, 345-347.

Lesk, A. M., & Chothia, C. (1984) *J. Mol. Biol. 174*, 175-191.

Markland, F. S., Bacharach, A. D. F., Weber, B. H., O'Grady, T. C., Saunders, G. C., & Umemura, N. (1975) *J. Biol. Chem. 250*, 1301-1310.

Mas, M. T., Chen, C. Y., Hitzeman, R. A., & Riggs, A. D. (1986) *Science (Washington, D.C.) 233*, 788-790.

Mas, M. T., Resplandor, Z. E., & Riggs, A. D. (1987) *Biochemistry 26*, 5369-5377.

Mori, N., Singer-Sam, J., & Riggs, A. D. (1986) *FEBS Lett. 204*, 313-317.

Pickover, C. A., McKay, D. B., Engelman, D. M., & Steitz, T. A. (1979) *J. Biol. Chem. 254*, 11323-11329.

Roustan, C., Fattoum, A., Jeanneau, R., & Pradel, L.-A. (1980) *Biochemistry 19*, 5168-5175.

Sanger, R., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.

Scopes, R. K. (1978) *Eur. J. Biochem. 85*, 503-516.

Stinson, R. A. (1974) *Biochemistry 13*, 4523-4529.

Tanswell, P., Westhead, E. W., & Williams, R. J. P. (1976) *Eur. J. Biochem. 63*, 249-262.

Watson, H. C., Walker, N. P. C., Shaw, P. J., Bryant, T. N., Wendell, P. L., Fothergill, L. A., Perkins, R. E., Conroy, S. C., Dobson, M. J., Tuite, M. F., Kingsman, A. J., & Kingsman, S. M. (1982) *EMBO J. 1*, 1635-1640.

Wilson, C. A. B., Hardman, N., Fothergill-Gilmore, L. A., Gamblin, S. J., & Watson, H. C. (1987) *Biochem. J. 241*, 609-614.

p10, a Low Molecular Weight Single-Stranded Nucleic Acid Binding Protein of Murine Leukemia Retroviruses, Shows Stacking Interactions of Its Single Tryptophan Residue with Nucleotide Bases†

Jose R. Casas-Finet, Nam-In Jhon, and August H. Maki*

Department of Chemistry, University of California, Davis, Davis, California 95616

Received August 19, 1987; Revised Manuscript Received October 23, 1987

ABSTRACT: Room temperature fluorescence and low-temperature phosphorescence studies of the association of p10, a basic low molecular weight single-stranded DNA binding protein isolated from murine leukemia viruses, point to the involvement of its single tryptophan residue in a close-range interaction with single-stranded polynucleotides. Optically detected triplet-state magnetic resonance (ODMR) techniques applied to the complex of p10 protein with the heavy atom derivatized polynucleotide poly(5-HgU) demonstrate the occurrence of stacking interactions of Trp^{35} with nucleic acid bases, thus agreeing with earlier reports that this residue is involved in the binding process [Karpel, R. L., Henderson, L. E., & Oroszlan, S. (1987) *J. Biol. Chem. 262*, 4961-4967].

Most retroviruses can be distinguished by their pathogenicity [for a review, see Bishop (1978)]. Many are oncogenic (Oncovirinae) and are commonly identified according to the type of neoplasm they induce and the species from which the virus has been isolated (Bishop, 1978). Given the pathogenic role of these viruses, considerable effort has been directed to obtain a better understanding of their biology, which may suggest therapeutic and preventive measures against infection. Genomes of endogenous retroviruses reside in the germ lines of many, if not all, species and segregate as normal genetic elements within these species (Bishop, 1978). Both endogenous (baboon) and exogenous (bovine, feline) retroviruses are known to induce enzootic leukosis (lymphosarcoma, leukemia) in their respective hosts (Burny et al., 1980; Essex, 1980). Bovine leukemia virus (BLV) infects 20% of all domesticated cattle in the United States (Ferrer, 1979); infectious virus has been demonstrated in milk of dairy cows (Ferrer et al., 1981) and has proven infectious in other species including chimpanzee (Van Der Maaten & Miller, 1976). Feline leukemia virus (FLV) infects most free-roaming cats and is responsible for

† This research was supported by a grant from the National Institutes of Health, ES-02662 (A.H.M.).

* Author to whom correspondence should be addressed.

0006-2960/88/0427-1172$01.50/0

one-third of all cancer deaths in this population (Essex, 1980). Moreover, retroviruses etiologically associated with lymphotrophic and immunosuppressive diseases in simians and humans have been discovered. HTLV-I, -II, and -III viruses have been identified as the causative agents of human adult T-cell leukemia (Poiesz et al., 1980; Yoshida et al., 1982; Weiss, 1982) and acquired immune deficiency syndrome (AIDS) (Popovic et al., 1984). Cytopathic retroviruses are also linked to lentiviral infections, which induce chronic degenerative diseases such as equine infectious anemia, caprine arthritis-encephalitis, and possibly human senile dementia. Some retroviruses are symbiotic and evoke little or no response in the host cell (Bishop, 1978).

Replication-competent mammalian and avian retroviruses display an integrated proviral DNA which is collinear with the viral genomic RNA (Bishop, 1978). The proviral genome contains three structural genes flanked by long terminal repeats (LTR) arranged in the order 5′-LTR-*gag*-*pol*-*env*-LTR-3′ (Coffin, 1982). While the *pol* gene encodes an RNA-dependent DNA polymerase and the *env* gene product is a precursor of one of the viral envelope proteins (Baltimore, 1974), the *gag* (group-specific antigen) gene encodes a polyprotein precursor which during virion maturation is processed into several fragments found in the core of the mature virus particle (Barbacid et al., 1976). The nucleotide and amino acid sequences of the *gag* and *pol* genes of different retroviruses exhibit considerable interspecific homology, indicating that they code for functionally important products which have been conserved throughout long evolutionary periods (Tamura, 1983; Sagata et al., 1985).

It has been shown that, among the 5′-coding regions of retroviruses, there is usually a very high antigenic and structural homology between a basic nucleic acid binding protein of low molecular weight which is a cleavage product of the *gag* precursor (Barbacid et al., 1976; Henderson et al., 1980; Morgan et al., 1983; Copeland et al., 1984; Ratner et al., 1985; Stephens et al., 1986; Berg et al., 1986). Furthermore, the nucleic acid binding proteins of bovine leukemia virus (Copeland et al., 1983a), human T-cell leukemia virus (Copeland et al., 1983b), AIDS virus (Ratner et al., 1985), visna lentivirus (Sonigo et al., 1985), equine infectious anemia virus (Stephens et al., 1986), and Rous-avian sarcoma virus (Misono et al., 1980) contain an internal repeat which may have evolved through a process of partial gene duplication (Henderson et al., 1980). In the case of the nucleic acid binding protein from Rous sarcoma virus, amino- and carboxy-terminal fragments containing the putative DNA binding domains have been produced by CNBr cleavage; each of these fragments contains a conserved structure of three cysteine residues and one histidine residue and bind independently to RNA (Misono et al., 1980). A similar but inverted set of three Cys and one His is present in one of the best characterized single-stranded DNA binding proteins, the gene 32 protein (gp32) from bacteriophage T4 (Williams et al., 1981). It has been proven recently that gp32 is a metalloprotein which contains one Zn atom per molecule complexed to such residues (Giedroc et al., 1986). The existence of metal binding domains in other nucleic acid binding proteins including those of retroviruses has been suggested (Berg et al., 1986).

Type C murine and feline retroviruses code for a nucleic acid binding protein which contains only one of such putative DNA and metal binding domains (Henderson et al., 1981; Copeland et al., 1984; Berg et al., 1986). The p10 protein from Rauscher murine leukemia virus (R-MuLV) is a linear polypeptide of 56 residues (M_r 6347) of known sequence (Henderson et al., 1981). It contains one Trp (at position 35), one Tyr (at position 28), no Phe, three Cys (at positions 26, 29, and 39), one His (at position 34), and an excess of basic residues. p10 binds nonspecifically and preferentially to single-stranded RNA and DNA (Davis et al., 1976). Selective chemical modification suggests that Tyr and Lys residues may be involved in the binding to nucleic acids (Henderson et al., 1980). The involvement of Tyr and/or Trp in hydrophobic (stacking) interactions with nucleic acid bases is suggested by the fact that binding to ssDNA still occurs at pH 10, when p10 should have a net negative charge (its isoelectric point is 9.5) (Davis et al., 1976). p10 is therefore one of the smallest known nucleic acid binding proteins and should constitute a good model system for studies of protein–nucleic acid interaction. We present evidence in this study for the involvement of the single Trp residue of p10 in stacking interactions with polynucleotides.

Materials and Methods

The p10 protein was isolated from virus suspensions by extraction with organic solvent (Olpin & Oroszlan, 1980) and purified by ion-exchange chromatography on phosphocellulose columns (Whatman P11) as described previously (Henderson et al., 1981). The purity of the protein was established by Coomassie Brilliant Blue stained sodium dodecyl sulfate (SDS)–polyacrylamide (15%) gel electrophoresis according to the method of Laemmli (1970). The protein concentration was determined by amino acid analysis.

The apparatus and experimental procedures for measurements of phosphorescence spectra and lifetimes, slow- and fast-passage ODMR[1] responses, and wavelength-selected ODMR have been described in detail previously [see Khamis et al. (1987) and references cited therein].

Fluorometric measurements monitoring the intrinsic protein fluorescence of p10 were conducted similarly to those described by Khamis et al. (1987). Analysis of the equilibrium binding isotherms and salt-back titrations was carried out by standard procedures (Kelly et al., 1976; Williams et al., 1983).

Results and Discussion

Biochemical Characterization of Protein Samples. Three bands were found on SDS–polyacrylamide gels upon electrophoresis of p10 samples, and the apparent molecular weight of the species was calculated by comparison with a set of protein standards. An intense band at 13 kDa was detected together with a fainter band at ca. 10 kDa and a very weak band at 18 kDa (data not shown). These bands were assigned to dimeric, monomeric, and trimeric forms of the protein, respectively. Evidence supporting this assignment will be given below.

The amino acid composition of the protein samples was in excellent agreement with the published sequence (Henderson et al., 1981). This rules out the occurrence of contamination by other proteins. p10 was run through two HPLC gel filtration columns (Whatman I125) placed in tandem. Two closely spaced peaks were found (data not shown), which can be assigned to monomeric and dimeric forms of the protein. Although the molecular weight of these species (calculated by calibration with a set of protein standards) is somewhat higher than the expected molecular weight, it is known that chromatographic methods result in an overestimation of the

[1] Abbreviations: AM-PMDR, amplitude-modulated phosphorescence microwave double resonance; $|D|$ and $|E|$, triplet-state zero field splitting parameters; ODMR, optically detected magnetic resonance; poly(dT), poly(thymidylic acid); poly(5-HgU), mercurated poly(uridylic acid).

p10 apparent size (Nowinski et al., 1972). The stability of the dimers, eluted in a phase containing 0.1% (v/v) trifluoroacetic acid, 6 M guanidine hydrochloride, and 40% acetonitrile, seems to rule out the possibility of noncovalent association. Formation of disulfide bridges by the cysteine residues of p10 could explain the observed intermolecular cross-linking. Another possibility is the existence of a population heterogeneity of p10 molecules with different molecular weights. Since p10 is generated by cleavage of the gag polyprotein by a viral proteinase, the occurrence of different proteolytic processing sites could create a set of p10 proteins of different size. This would be noticed, however, by the lack of agreement of the p10 amino acid composition with the published sequence. It is also known that the N- and C-terminal residues of several nucleic acid binding proteins of retroviruses are identical, a fact that may reflect the conservation of proteolytic cleavage sites among different species (Copeland et al., 1984). However, a p10 protein that is an incomplete *gag* cleagave product and contains four additional amino acid residues at its COOH terminus has been reported in the literature (Henderson et al., 1984). Although such heterogeneity is not ruled out, it cannot explain the much larger difference in molecular weight observed in our gels. Furthermore, when the protein samples were heated in sealed vials at 100 °C for 5 min, in a buffer containing 10 mM 2-mercaptoethanol and 1 mM dithiothreitol, only one band appeared in the gel electrophoresis experiments. This band migrated faster than the smallest protein standard of the electrophoresis calibration kit (cytochrome *c*, M_r 11.5K). This demonstrates the purity of our protein sample and suggests that an excess of these thiol-protecting reagents should be maintained in the samples to keep the cysteine residues of p10 in the reduced form. It is worth mentioning that preliminary high-field (500-MHz) ^{1}H NMR studies on the p10 protein (Casas-Finet, 1985) showed considerable broadening of the resonances in the aromatic region unless the sample is incubated at 40 °C for 12 h in the presence of 20 mM dithiothreitol. The longer rotational correlation times expected for the oligomeric structures involving p10–p10 cross-linking at their Cys residues could account for the observed effect. The chemical shift of the aromatic residues also is affected by the redox state of the p10 Cys residues (Casas-Finet, 1985). Each of the aromatic residues of p10 is included in a region enclosed by the residues Cys^{26} and Cys^{38} (Henderson et al., 1981). The proposed involvement of three Cys and one His in the complexation of one Zn atom is consistent with the downfield shifts of the NMR signals from the H(2) and H(4) protons of histidine upon reductive treatment of the p10 Cys residues (Casas-Finet, 1985). These results suggest that the luminescence properties of the tryptophan chromophores also may be affected by the redox state of p10.

Fluorescence Titrations. p10 displays a fluorescence emission spectrum characteristic of a tryptophan chromophore, with a maximum at 353 nm. This relatively red-shifted fluorescence suggests that the single Trp residue of p10 is in a hydrophilic environment (probably solvent exposed in a protein of such small size). No contribution of Tyr fluorescence (maximum at ca. 310 nm) to the intrinsic fluorescence could be detected (excitation wavelength = 282 nm), after subtraction of the Raman scattering peak from the solvent. This points either to a small fluorescence quantum yield of Tyr^{28} or to an efficient energy-transfer mechanism to Trp^{35}.

The intrinsic fluorescence emission of p10 is quenched upon binding to single-stranded polynucleotides (Casas-Finet, 1985; Karpel et al., 1987). Analysis of the fluorometric binding

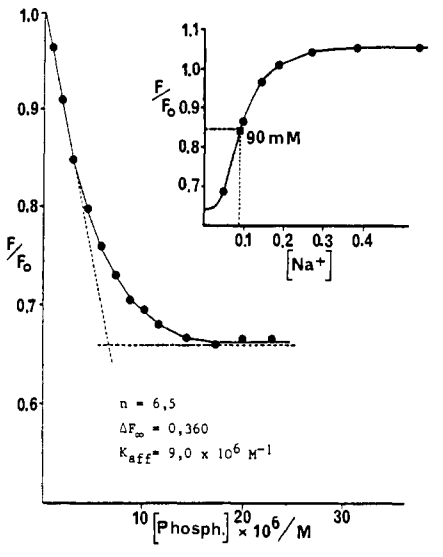

FIGURE 1: Fluorometric binding isotherm of the association of p10 (1×10^{-6} M) to poly(dT) (concentration in moles of nucleotide bases) at 25 °C in a 50 mM NaH_2PO_4/Na_2HPO_4 (pH 7.2) buffer. Measurements were made at 350 nm with excitation at 282 nm. (Insert) a salt-back titration of the p10/poly(dT) complex obtained by addition of aliquots of 5 M NaCl solution.

isotherms (Figure 1) obtained by stepwise addition of poly(dT) allows us to determine the binding site size (n), the limiting fluorescence quenching (Q_{max}), and the affinity constant (K_{aff}). The occluded binding site size of 6.5 nucleotides/bound protein (calculated from extrapolation of the initial slope and plateau regions of the fluorometric titration) is in excellent agreement with the end point of similar titrations of p10 with poly(U) or of the fluorescent polynucleotide poly(A) with p10 ($n = 6 \pm 1$) (Karpel et al., 1987). The limiting quenching, $Q_{max} = 0.36$, is smaller than the value found for poly(U) ($Q_{max} = 0.68$) (Karpel et al., 1987). In addition, the affinity of p10 for poly(dT) at the ionic strength used in our experiments is about 1 and 2 orders of magnitude higher than the one found for poly(ϵA) and poly(U), respectively, under similar ionic conditions (Karpel et al., 1987). Poly(dT) also is the polynucleotide bound most tightly by other single-stranded DNA binding proteins such as gene 32 protein from bacteriophage T4 and SSB protein from *Escherichia coli* [for a review, see Chase and Williams (1986)] due to its lack of secondary structure. Note the low value of the salt-back midpoint (90 mM, Figure 1 insert), which is of a magnitude comparable to that previously reported for other polynucleotides (Karpel et al., 1987).

Addition of excess 2-mercaptoethanol to the buffer yields nearly identical titration curves (data not shown). Therefore, binding of p10 to polynucleotides is independent of the redox state of the Cys residues. At the protein concentration (ca. 10^{-4} M) and ionic strength (ca. 20 mM) used in our ODMR experiments, quantitative binding of p10 to poly(dT) and poly(5-HgU) will result [assuming an affinity of p10 for poly(5-HgU) similar to that reported for poly(U) by Karpel et al. (1987)].

Phosphorescence Spectra and Lifetime. The phosphorescence spectrum of p10 protein incubated in the presence of a 100-fold excess of 2-mercaptoethanol is shown in Figure 2A. The resolved 0,0-band peaks at 410.5 ± 1 nm (Table I). One can also detect a contribution of tyrosine phosphorescence at $\lambda < 400$ nm, which is to the blue of the onset of the tryptophan phosphorescence (Figure 2A). In the absence of incubation with sufficient 2-mercaptoethanol, p10 exhibits a broader phosphorescence 0,0-band which peaks at 412.0 ± 2 nm

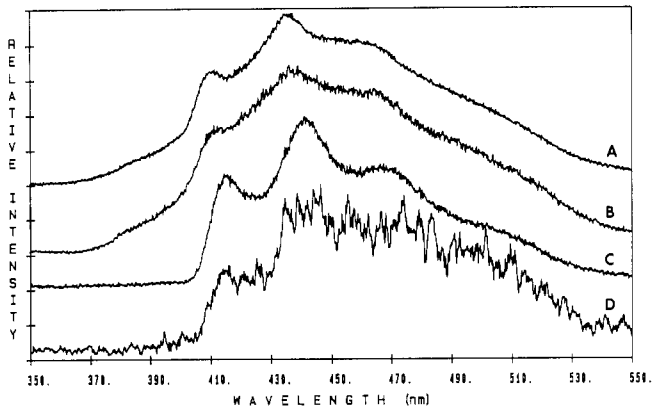

FIGURE 2: Phosphorescence spectra of (A) reduced p10 (4.0×10^{-4} M), (B) oxidized p10 (4×10^{-4} M), and (C) reduced p10 (1.2×10^{-4} M) complexed with poly(5-HgU) (1.35×10^{-3} M) and (D) AM-PMDR spectrum of reduced p10 (1.2×10^{-4} M) complexed with poly(5-HgU) (1.35×10^{-3} M). Excitation was at 295 nm with 16-nm band-pass, and the emission slits were set at 3-nm resolution. Temperature was 77 K for (A)–(C), and 1.2 K for (D). Spectrum D was obtained by amplitude modulation of microwaves at 50 Hz; they also were frequency modulated at 5.0 KHz over a range of ±500 MHz about the center resonance frequency of 4.43 GHz. Phase-sensitive detection was employed at 50 Hz. Signal averaging was carried out for eight scans. The samples were prepared in 20 mM cacodylate buffer, pH 7.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 15 mM 2-mercaptoethanol, 30% glycerol, and ca. 20 mM NaCl, except for (B) which contains 0.1 mM 2-mercaptoethanol.

Table I: Phosphorescence Lifetimes of Tryptophan in p10 and Its Complexes with Various Single-Stranded Polynucleotides[a]

sample	$\lambda_{0,0}$ (nm)	lifetime components (s)[b]
p10 reduced	410.5	5.48 (59%), 0.63 (41%)
p10 oxidized	412.0[c]	4.15 (39%), 0.56 (61%)
p10 reduced + poly(dT)	412.8	4.85 (42%), 0.78 (58%)
p10 oxidized + poly(dT)	415.0	4.12 (49%), 0.42 (51%)
p10 + poly(5-HgU)	416.0	0.077 (25%), 0.006 (75%)

[a] Measurements were made at 77 K, the excitation wavelength was 295 nm, and the emission was monitored at the 0,0-band peak as indicated with the monochromator set at 3-nm band-pass. The error in the tabulated wavelength of the phosphorescence maxima is ±1 nm, unless indicated otherwise. The phosphorescence decay was fit to two exponential components with the $1/e$ lifetimes and preexponential contributions given. [b] The phosphorescence decays were not simple. Deconvolution by computer was limited arbitrarily to two exponential components which provide a satisfactory approximation of the decay profile. [c] The error is ca. ±2 nm.

(Figure 2B, Table I). Upon binding to poly(dT), both samples display a red shift of the phosphorescence maxima (Table I) to 412.8 and 415.0 nm for the reduced and oxidized form of p10, respectively. This confirms our fluorometric results, which indicate that both forms of p10 are able to bind to poly(dT).

Binding p10 to poly(5-HgU) induces the largest red shift of the 0,0-band (Figure 2C), which peaks at 416.0 nm (Table I). A Trp phosphorescence red shift is known to result from the lower energy of the Trp residues which undergo stacking with nucleic acid bases (Rahn et al., 1966). Further evidence for stacking as the cause of the observed red shift will be presented in the AM-PMDR section. The vibronic structure of the Trp phosphorescence spectrum of the p10/poly(5-HgU) complex is more resolved than that of the free protein (compare spectra C and A of Figure 2), indicating a more homogeneous environment of the Trp chromophore. The phosphorescence emission is also more intense in the complex than in the free protein, a fact that can be explained by increased intersystem crossing to be expected from a heavy atom effect of mercury on the indole luminescence. On the other hand, the phosphorescence emission from tyrosine is now missing (compare

Table II: Tryptophan Zero Field ODMR Frequencies and Zero Field Splitting Parameters in p10 and Its Complexes with Various Single-Stranded Polynucleotides[a]

sample	$\lvert D\rvert - \lvert E\rvert$ (GHz)	$2\lvert E\rvert$ (GHz)	$\lvert D\rvert + \lvert E\rvert$ (GHz)	$\lvert D\rvert$ (GHz)	$\lvert E\rvert$ (GHz)
p10 reduced	1.76	2.54	*b*	3.03	1.27
p10 oxidized	1.75	2.60	*b*	3.05	1.30
p10 reduced + poly(dT)	1.74	2.63	*b*	3.06	1.32
p10 oxidized + poly(dT)	1.74	2.62	*b*	3.05	1.31
p10 + poly(5-HgU)[c]	1.73	2.54	4.31	3.01	1.27

[a] ODMR measurements were made at 1.2 K with the emission monochromator at 3-nm resolution and set at the peak of the phosphorescence 0,0-band. Transition frequencies are corrected for rapid-passage effects. Esimated uncertainty in the ODMR frequencies is ±0.01 GHz. Microwaves were swept at 58 MHz s^{-1} unless otherwise indicated. [b] Signal is not observed. [c] Slow-passage ODMR signals were collected under rapid microwave sweep conditions (5.8 GHz s^{-1} for the $\lvert D\rvert - \lvert E\rvert$ and $2\lvert E\rvert$ signals; 20 GHz s^{-1} for the $\lvert D\rvert + \lvert E\rvert$ signal).

spectra A and C of Figure 2). Stacking of tyrosine residues with nucleic acid bases results in almost total quenching of the tyrosine phosphorescence emission due to an energy-transfer mechanism (J. R. Casas-Finet, M. I. Khamis, and A. H. Maki, unpublished results). These results suggest the involvement of both the Trp and Tyr residues of p10 in stacking interactions with single-stranded polynucleotides.

The longest component of the p10 multiexponential phosphorescence decay profile can be attributed to the contribution of Trp residues. The lifetime of this component is reduced from 5.48 s for the free (reduced) protein to 4.12 s in the oxidized form. Binding to poly(dT) induces a significant reduction of the phosphorescence lifetime of the Trp component only when p10 is in its reduced form (Table I). The largest effect is observed upon the binding of (reduced) p10 to poly(5-HgU) (Table I), resulting in the appearance of a component of less than 10 ms which dominates the decay profile (75%). It is known that an external heavy atom effect induces an enhancement of both the triplet-state radiative decay rate and the intersystem crossing rate to the ground singlet state. A lifetime reduction by 3 orders of magnitude is typical of a heavy atom perturbation by mercury in van der Waals contact with Trp (Anderson & Maki, 1970). Similar phosphorescence lifetimes are observed for Trp–CH_3Hg^+ complexes in which the mercury atom is known to be located above the indole plane. An edge-on perturbation by the mercury atom results in triplet-state lifetimes which are an order of magnitude greater than those which are found for an above-the-plane approach (Svejda et al., 1978). Moreover, because the Hg atom of 5-HgU is blocked by 2-mercaptoethanol, steric considerations limit the possibilities for a van der Waals contact between the heavy atom and the indole π-electrons to a stacking interaction. The minor longer component in the decay (ca. 77 ms) may reflect some disorder in the polynucleotide complexes resulting from the fact that the poly(5-HgU) is only 70% mercurated.

ODMR Spectra. The ODMR frequencies of the single Trp residue of p10 and its polynucleotide complexes are presented in Table II. Although differing in the proteins themselves, the values for the poly(dT) complexes of p10 in its reduced and oxidized state are indistinguishable within the accuracy of the measurements (±0.01 GHz). This result indicates that the differences in the tryptophan microenvironment (e.g., the redox state of the neighboring cysteine residues) are more apparent in the case of the free proteins than for the p10/poly(dT) complex, as suggested by the phosphorescence spectra and lifetime results. In other words, binding to poly(dT)

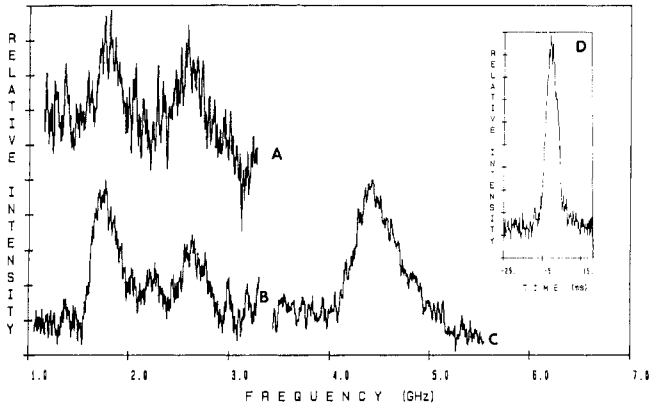

FIGURE 3: (A) Slow-passage $|D| - |E|$ (lower frequency) and $2|E|$ (higher frequency) ODMR transitions of Trp in p10 (4.0×10^{-4} M). The sweep rate was 58 MHz s^{-1}. Signal averaging was performed for 24 scans. The temperature was 1.2 K, and the phosphorescence was excited at 295 nm with 16-nm band-pass and monitored at 409.5 nm with 3-nm band-pass. (B) Slow-passage $|D| - |E|$ and $2|E|$ ODMR signals of heavy atom perturbed Trp in the p10/poly(5-HgU) complex 1.2×10^{-4} M:1.33×10^{-3} M). The sweep rate was 2.2 GHz s^{-1}, and 550 scans were signal averaged. Other conditions were as given in (A) except that the phosphorescence emission was monitored at 415 nm. (C) Slow-passage $|D| + |E|$ signal for the p10/poly(5-HgU) complex. The signal is the result of 4500 scans acquired at a sweep rate of 20 GHz s^{-1}. (D) Fast-passage response of the perturbed Trp in the p10/poly(5-HgU) complex obtained in the $|D| + |E|$ region. The microwaves were swept from 4.1 to 5.1 GHz at 100 GHz s^{-1}.

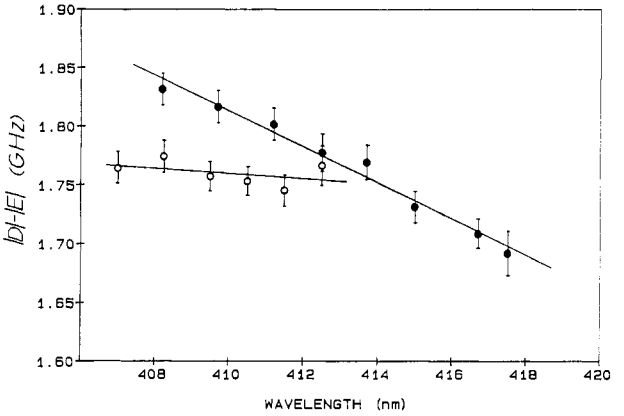

FIGURE 4: Plot of the $|D| - |E|$ ODMR frequency vs emission wavelength through the Trp 0,0-band region in p10 (open circles) and in the p10/poly(5-HgU) complex (solid circles). The microwave sweep rate was 58 MHz s^{-1} (open circles) and 10 GHz s^{-1} (solid circles). Transition frequencies were corrected for rapid-passage effects. Excitation wavelength was 295 nm with 16-nm band-pass. Emission slits were set at 1.5-nm band-pass, and the sample temperature was 1.2 K. Error bars represent ±5% FWHM (full width at half-maximum) of the peak.

induces a perturbation of the Trp microenvironment which dominates over other contributions. This would be the expected effect for a close-range interaction of Trp with the polynucleotide. Although a stacking interaction with the thymine bases of poly(dT) would be expected to lead to a decrease of the $|D|$ parameter due to electronic delocalization along the z (stacking) axis, a small increase is observed instead [compare the zero field splittings in Table II for the reduced form of p10, free and bound to poly(dT)]. However, the longest predicted α-helical domain of p10 (see below), which among its 13 amino acid residues contains all the aromatic and Cys residues, also contains six charged residues; these polar residues could induce electric field (Stark) effects on Trp upon binding of p10 to poly(dT) and therefore compensate for the expected reduction in $|D|$ due to stacking. The polyphosphate backbone of the polynucleotide also could induce such effects. In this respect, it is worth mentioning that the increase in the zero field splitting parameters observed when reduced p10 is complexed with poly(dT), relative to the free protein (Table II), is larger for $|E|$ (50 MHz) than it is for $|D|$ (30 MHz); $|E|$ values are affected mainly by changes in the environment of the indole plane, while changes in $|D|$ may be related to environmental changes occurring along the z (stacking) axis.

The most convincing evidence for the presence of stacking interactions is given by the p10/poly(5-HgU) complex. The Trp $|D| - |E|$ signals of p10 (Figure 3A, Table II) are shifted to lower frequencies upon complexation to poly(5-HgU) (Figure 3B, Table II). The ODMR signals of the p10/poly(5-HgU) complex are collected at a rapid microwave sweep rate, which allows us to monitor selectively the heavy atom perturbed Trp residues of p10. Under these conditions the slow-passage signals are somewhat distorted by rapid-passage transient effects. The true peak position of the ODMR transitions was obtained, however, by extrapolation of the measured frequencies at different microwave sweep rates to zero sweep rate.

Most notably, the p10/poly(5-HgU) complex displays the normally unobservable $|D| + |E|$ transition (Figure 3C, Table II). A transient microwave fast-passage experiment through this transition produces a lifetime of ca. 3 ms, which can be assigned to the more radiative sublevel of the transition pair (Figure 3D). These effects are diagnostic of an above-the-plane (stacking) approach of the heavy atom derivatized uracil base, which introduces a spin–orbit coupling mechanism that greatly enhances the rates of multiplicity-forbidden processes.

AM-PMDR Spectrum of p10 Complex with Poly(5-HgU). The phosphorescence spectrum of the heavy atom perturbed triplet state may be obtained selectively by using the amplitude-modulated phosphorescence microwave double resonance (AM-PMDR) method (Olmstead & El-Sayed, 1974). Figure 2D shows the AM-PMDR spectrum acquired for the p10/poly(5-HgU) complex by amplitude modulating the Trp $|D| + |E|$ transition and using lock-in detection at the modulation frequency. The 0,0-band of the Hg-perturbed Trp obtained by AM-PMDR peaks at 415.2 nm, in good agreement with the tabulated value of the phosphorescence 0,0-band of the poly(5-HgU) complex, 416.0 nm (Table I). This shows that the Trp phosphorescence spectrum of the p10/poly(5-HgU) complex originates from the heavy atom perturbed chromophores. The broadness of the AM-PMDR spectrum relative to the phosphorescence may be justified on the basis of the enhancement of vibronic intensity of low-frequency modes induced by Hg (Davis & Maki, 1982). The low-frequency vibronic intensity could appear selectively in the AM-PMDR rather than the phosphorescence, resulting in reduced resolution.

Wavelength-Selected ODMR. Wavelength-selected ODMR experiments in which the zero field splittings are monitored throughout the phosphorescence 0,0-band with narrow slits can reveal the presence of more than one type of Trp site if discontinuities occur. As shown in Figure 4, both the free reduced p10 protein (open circles) and the p10/poly(5-HgU) complex (full circles) reveal only a single type of resolvable Trp microenvironment. However, the Trp site of the free protein which has nearly wavelength-independent zero field splittings is transformed to a wavelength-dependent site upon binding to poly(5-HgU) (see Figure 4). We also notice that, although little change is observed when the phosphorescence is monitored at the respective 0,0-band peak wavelengths, there is a significant increase of the $|D| - |E|$ transition frequency at shorter emission wavelengths as a result of complex for-

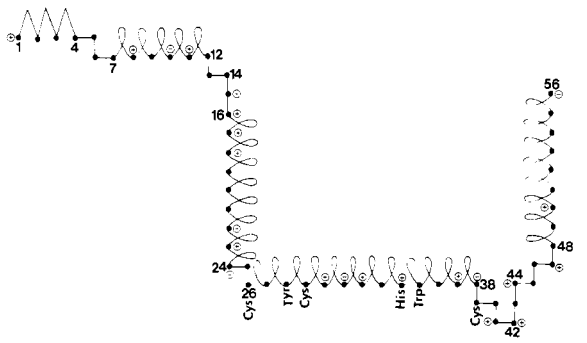

FIGURE 5: Predicted secondary structure of p10 protein according to the method of Chou and Fasman (1978). Symbols denotate α-helix (ℓ), β-sheet ($\wedge$), β-turn ($\urcorner$), and random coil (—) structure. The positions of aromatic, Cys, and charged residues in the sequence are indicated.

mation. An increase in the $|D| - |E|$ transition frequency of Trp has been detected previously for the Trp–methylmercury complex (Svejda et al., 1978) and for the poly(5-HgU) complexes of *E. coli* SSB (Khamis et al., 1987a), the *E. coli* plasmid-encoded pIP231a SSB (J. R. Casas-Finet, N.-I. Jhon, M. I. Khamis, P. P. Ruvolo, and J. W. Chase, unpublished results), and gene 32 protein (J. R. Casas-Finet, J.-J. Toulme, R. Santus, and A. H. Maki, unpublished results). This frequency shift has been attributed to a spin–orbit coupling and/or Coulombic perturbation that counteracts the expected stacking-induced decrease of the $|D|$ zero field splitting parameter.

CONCLUSIONS

p10 reveals a significant quenching of fluorescence and a phosphorescence red shift upon association with thymine polynucleotides, suggesting the involvement of its single Trp residue in the binding process. Binding of poly(dT) induces a significant reduction of the Trp phosphorescence lifetime when p10 cysteine residues are in the free thiol form. Biochemical and physicochemical evidence suggests that the formation of protein dimers depends on the redox state of the p10 cysteine residues. Although oxidized p10 shows spectral differences with the reduced form of the protein which can be attributed to changes in the Trp microenvironment, both forms bind to poly(dT). The association of p10 with the heavy atom derivatized homopolymer poly(5-HgU) induces a dramatic decrease of 3 orders of magnitude in the phosphorescence lifetime of Trp^{35}. An effect of this magnitude is known to originate from a close-range above-the-plane approach of the Hg atom within van der Waals distance of the indole π-orbitals. Detection of the normally dark $|D| + |E|$ ODMR signal and analysis of the fast-passage response induced by this transition, which exhibits a lifetime of less than 3 ms, confirm the existence of an extremely efficient external heavy atom effect. Further evidence is provided by the AM-PMDR spectrum. Steric considerations lead to the conclusion that aromatic stacking interactions of Trp with the nucleotide bases are present.

Secondary structure prediction by the method of Chou and Fasman (1978) suggests that residues 26–39 form an α-helix (Figure 5). Model building shows that the aromatic rings of Tyr^{28} and Trp^{35} can be oriented to intercalate between nucleotide bases of trinucleotides (Henderson et al., 1980). The central base would then be in close contact with Glu^{31} and Lys^{32} (Henderson et al., 1980). Chemical modification suggests the involvement of Tyr and Lys residues of p10 in the nucleic acid binding site (Henderson et al., 1980).

Residues Trp^{54} and Phe^{60} of *E. coli* SSB, a well-characterized prokaryotic single-stranded DNA binding protein, have been implicated in stacking interactions with nucleic acid bases (Khamis et al., 1987b,c; Casas-Finet et al., 1987). These residues, as well as Tyr^{28} and Trp^{35} of p10, would be separated by roughly two α-helix turns (ca. 10.8 Å apart); hence, they will be facing roughly the same direction and therefore superimposed in an axial view of the protein α-helical segment. In such an extended structure, however, it is difficult to visualize a possible conformation for the putative polydentate metal binding domain which is predicted to include residues Cys^{26}, Cys^{29}, His^{34}, and Cys^{39}. Chemical modification of the p10 Cys residues by S-carboxyamidomethylation yields a derivatized protein with retains binding to poly(ϵA) (Karpel et al., 1987) and results in comparable enhancement of the fluorescence of this polynucleotide. On the other hand, performic acid oxidation of the p10 Cys residues (a process which converts cysteine into cysteic acid, with the concomitant destruction of the tryptophan indole ring), although reported to result in a derivatized protein which can still bind to poly(ϵA) (Karpel et al., 1987), decreases the salt-back midpoint for the p10/poly(ϵA) complex from 130 to 90 mM NaCl [compare Figures 3 and 7 of Karpel et al. (1987)]. Furthermore, the extent of fluorescence enhancement of poly(ϵA) induced by binding of p10 changes from 4.8- to 2.8-fold at saturation (Karpel et al., 1987). Since the fluorescence of this polynucleotide increases as a result of base unstacking, these results suggest that the Trp residue is involved in stacking with the nucleotide bases, as is demonstrated in our study, but it may not be the only aromatic residue involved in such interactions. Given the amino acid composition of p10, the only likely candidate is Tyr^{28}. Our results provide only indirect evidence for the participation of Tyr^{28} in the binding process. Luminescence studies are hampered by the weak emission of Tyr in the presence of a nearby Trp. The presence of a stoichiometrically bound metal atom in the p10 protein has not yet been demonstrated. Further studies are needed to address these questions.

Registry No. Poly(dT), 25086-81-1; poly(U), 27416-86-0; L-Trp, 73-22-3.

REFERENCES

Anderson, R. R., & Maki, A. H. (1980) *J. Am. Chem. Soc. 102*, 163–167.

Baltimore, D. (1974) *Cold Spring Harbor Symp. Quant. Biol. 39*, 1187–1200.

Barbacid, M., Stephenson, J. R., & Aaronson, S. A. (1976) *J. Biol. Chem. 251*, 4859–4866.

Berg, J. M. (1986) *Science (Washington, D.C.) 232*, 485–487.

Bishop, J. M. (1978) *Annu. Rev. Biochem. 47*, 35–88.

Burny, A., Bruck, C., Chantrenne, H., Cleuter, Y., Dekegel, D., Ghysdael, J., Kettmann, R., Leclercq, M., Leunen, J., Mammerick, M., & Portetelle, D. (1980) in *Viral Oncology* (Klein, G., Ed.) pp 231–289, Raven, New York.

Casas-Finet, J. R. (1985) These Docteur es-Sciences Physiques, University of Paris VI, France.

Casas-Finet, J. R., Khamis, M. I., Maki, A. H., & Chase, J. W. (1987) *FEBS Lett. 220*, 347–352.

Chase, J. W., & Williams, K. R. (1986) *Annu. Rev. Biochem. 55*, 103–136.

Coffin, J. (1982) in *RNA Tumor Viruses* (Weiss, R., Teich, N., Varmus, H., & Coffin, J., Eds.) pp 267–368, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Copeland, T. D., Morgan, M. A., & Oroszlan, S. (1983a) *FEBS Lett. 156*, 37–40.

Copeland, T. D., Oroszlan, S., Kalyanaraman, V. S., Sarngadharan, M. G., & Gallo, R. C. (1983b) *FEBS Lett. 162*, 390–395.

Copeland, T. D., Morgan, M. A., & Oroszlan, S. (1984) *Virology 133*, 137–145.
Davis, J., Scherer, M., Tsai, W. P., & Long, C. (1976) *J. Virol. 18*, 709–718.
Davis, J. M., & Maki, A. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4313–4316.
Essex, M. (1980) in *Viral Oncology* (Klein, G., Ed.) pp 205–229, Raven, New York.
Ferrer, J. F. (1979) *J. Am. Vet. Med. Assoc. 175*, 1281–1286.
Ferrer, J. F., Kenyon, S. J., & Gupta, P. (1981) *Science (Washington, D.C.) 213*, 1014–1016.
Giedroc, D. P., Keating, K. M., Williams, K. R., Konigsberg, W. H., & Coleman, J. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8452–8456.
Henderson, L. E., Long, C. W., & Oroszlan, S. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1606.
Henderson, L. E., Copeland, T. D., Sowder, R. C., Smythers, G. W., & Oroszlan, S. (1981) *J. Biol. Chem. 256*, 8400–8406.
Henderson, L. E., Sowder, R., Copeland, T. D., Smythers, G., & Oroszlan, S. (1984) *J. Virol. 52*, 492–500.
Karpel, R. L., Henderson, L. E., & Oroszlan, S. (1987) *J. Biol. Chem. 262*, 4961–4967.
Kelly, R. C., Jensen, D. E., & von Hippel, P. H. (1976) *J. Biol. Chem. 251*, 7240–7250.
Khamis, M. I., & Maki, A. H. (1986) *Biochemistry 25*, 5865–5872.
Khamis, M. I., Casas-Finet, J. R., Maki, A. H., Murphy, J. B., & Chase, J. W. (1987a) *FEBS Lett. 211*, 155–159.
Khamis, M. I., Casas-Finet, J. R., Maki, A. H., Ruvolo, P. P., & Chase, J. W. (1987b) *Biochemistry 26*, 3346–3354.
Khamis, M. I., Casas-Finet, J. R., Maki, A. H., Murphy, J. B., & Chase, J. W. (1987c) *J. Biol. Chem. 262*, 8574–8583.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Misono, K. S., Sharief, F., & Leis, J. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1611.
Morgan, M. A., Copeland, T. D., & Oroszlan, S. (1983) *J. Virol. 46*, 177–186.
Nissen-Meyer, J., & Abraham, A. K. (1980) *J. Mol. Biol. 142*, 19–28.
Nowinski, R. C., Fleissner, E., Sarkar, N. H., & Aoki, J. (1972) *J. Virol. 9*, 359–366.
Olmstead, J., & El-Sayed, M. A. (1974) in *Creation and Detection of the Excited State* (Ware, W. D., Ed.) pp 1–62, Dekker, New York.
Olpin, J. L., & Oroszlan, S. (1980) *Anal. Biochem. 103*, 331–336.
Poiesz, B. J., Rusetti, F. W., Gazdan, A. F., Bunn, P. A., Minna, J. D., & Gallo, R. C. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7415–7419.
Popovic, M., Sarngadharan, M. G., Read, E., & Gallo, R. C. (1984) *Science (Washington, D.C.) 224*, 497–500.
Rahn, R. O., Yamane, T., Eisinger, J., Longworth, J. W., & Shulman, R. G. (1966) *J. Chem. Phys. 45*, 2947–2954.
Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. R., Baumeister, K., Ivanoff, L., Petteway, S. R., Jr., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C., & Wong-Staal, F. (1985) *Nature (London) 313*, 277–284.
Sagata, N., Yasunaga, T., Ohishi, K., Tsuzuku-Kawamura, J., Onuma, M., & Ikawa, Y. (1984) *EMBO J. 19*, 3231–3237.
Sonigo, P., Alizon, M., Staskus, K., Klatzmann, D., Cole, S., Danos, O., Retzel, E., Tiollais, P., Haase, A., & Wain-Hobson, S. (1985) *Cell (Cambridge, Mass.) 42*, 369–382.
Stephens, R. M., Casey, J. W., & Rice, N. R. (1986) *Science (Washington, D.C.) 231*, 589–594.
Svejda, P., Maki, A. H., & Anderson, R. R. (1978) *J. Am. Chem. Soc. 100*, 7138–7145.
Tamura, T.-A. (1983) *J. Virol. 47*, 137–145.
Van Der Maaten, M. J., & Miller, J. M. (1976) *Vet. Microbiol. 1*, 351–357.
Williams, K. R., LoPresti, M. B., & Setoguchi, M. (1981) *J. Biol. Chem. 256*, 1754–1762.
Williams, K. R., Spicer, E. K., LoPresti, M. B., Guggenheimer, R. A., & Chase, J. W. (1983) *J. Biol. Chem. 258*, 3346–3355.
Yoshida, M., Miyoshi, I., & Hinuma, Y. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2031–2035.