Chromosoma (Berl.) 46, 255—260 (1974)

Fluorometric Properties of the Bibenzimidazole Derivative Hoechst 33258, a Fluorescent Probe Specific for AT Concentration in Chromosomal DNA

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Abstract. A new fluorescent probe of chromosomal DNA structure *in situ*, the bibenzimidazole derivative Hoechst 33258, shows enhanced fluorescence with both AT- and GC-rich DNA; however, enhancement by AT-rich DNA is greater than enhancement with GC-rich DNA. When this compound is used as a probe, it produces localized fluorescence which can be correlated with AT concentration in specific chromosome regions. By the use of 33258, Hilwig and Gropp (1972) were able to demonstrate the relatively AT-rich DNA present in centric regions of mouse chromosomes; these regions do not fluoresce brightly when treated with quinacrine because of the presence of guanine residues which are spaced with high periodicity and which therefore efficiently quench quinacrine fluorescence. The data obtained in this study with DNA polymers of defined structure or composition, as test model compounds, suggest that 33258 is a useful cytochemical reagent for generally identifying all types of AT-rich regions in chromosomes, including those which are not demonstrable with quinacrine.

Results and Discussion

In cytofluorometric studies of mouse chromosomes, Hilwig and Gropp (1972) reported that centric regions showed enhanced localized fluorescence after treatment with the bibenzimidazole derivative Hoechst 33258 (2-[2-(4-hydroxyphenyl)-6-benzimidalzolyl]-6-(1-methyl-4-piperazyl)-benzimidazole), while in an earlier characterization of the fluorometric properties of quinacrine-treated mouse chromosomes it was noted by Rowley and Bodmer (1971) that the centric regions showed a distinctive *lack* of fluorescence. Because of the opposite nature of the staining specificities of quinacrine and of bibenzimidazole 33258, we felt it would be of interest to determine which localized features of mouse chromosome structure were responsible for the fluorescence patterns observed, as well as to determine more generally, the chemical specificity of this new staining technique.

Our general approach to studying this type of problem is based on the fact that the fluorescence of certain dyes is modified by interaction with polynucleotides, *i.e.*, the dye can function as a *reporter* rather than

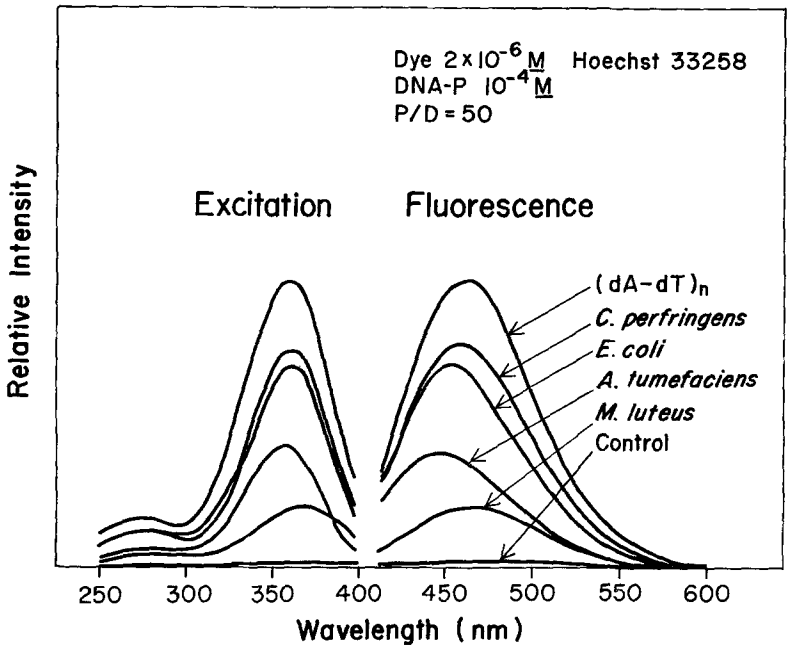

Fig. 1. Enhancement of bibenzimidazole (H 33258) fluorescence by DNA polymers. Fluorescence spectra of 2×10^{-6} *M* 33258 in 0.1 × SSC were determined in the presence of DNA polymers at a concentration of 10^{-4} *M* DNA phosphate. *C. perfringens* DNA (68% AT), *E. coli* DNA (49% AT), *M. luteus* DNA (27% AT) and $(dA\text{-}dT)_n$ were purchased from Sigma Chemical Co.; *A. tumefaciens* DNA (39% AT) was prepared by phenol extraction

purely as an affinity label, and it has been shown previously how the modification of acridine fluorescence depends on both the primary and secondary structure of the DNA as well as on neighboring base-pairs (Weisblum and de Haseth, 1972, 1973; Weisblum, 1973a, b). Moreover, these studies showed how data obtained in such experiments could be used to explain the cytological staining specificites of acridine dyes, notably quinacrine, proflavine, and acridine orange. This same approach has been used in connection with 33258.

Three experiments were performed. First, the fluorescence spectrum of 2×10^{-6} *M* 33258 was determined in the presence of a series of DNA polymers at the DNA-phosphate to dye ratio of 50 to 1. The results are shown in Fig. 1.

We note that the DNA polymers, ranging at AT composition from 0% to 68%, all enhance fluorescence in relation to their AT content and that this enhancement occurs at 465 nm. The bacterial DNA's were

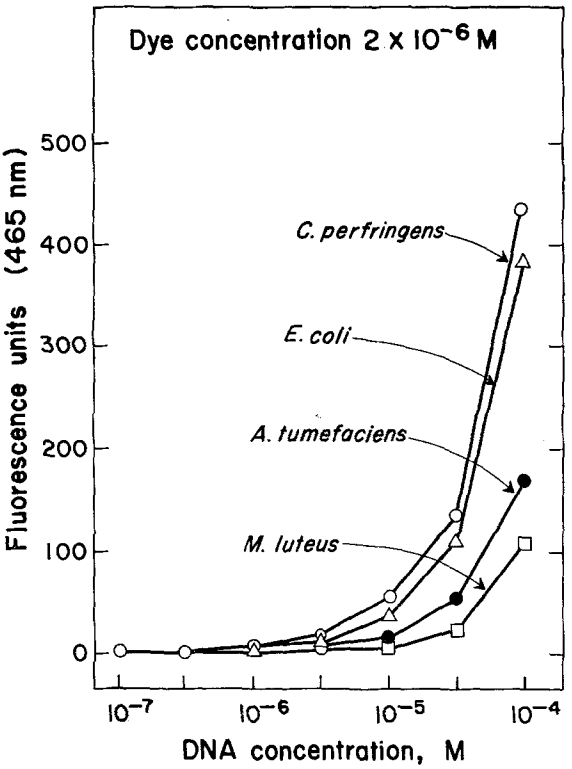

Fig. 2. Enhancement of bibenzimidazole (H 33258) fluorescence by bacterial DNA polymers as a function of DNA concentration. — The fluorescence of 2×10^{-6} *M* 33258 at 465 nm was determined as a function of increasing DNA concentration. Fluorescence was excited at 365 nm

from *Clostridium perfringens* (68% AT), *Escherichia coli* (49% AT), *Agrobacter tumefaciens* (39% AT) to *Micrococcus luteus* (27% AT). A shift to shorter wavelength of 10 nm, seen for *A. tumefaciens* DNA, can be regarded as insiginificant for the purposes of this study. We conclude that the cytofluorometric effects observed are due to enhancement of fluorescence at 465 nm rather than to possible shifts in the emission maximum.

Second, the concentration-dependence of fluorescence-enhancement at 465 nm was determined for a series of bacterial DNAs. The results are shown in Fig. 2. We note that over the range tested, the bacterial DNA's enhance fluorescence in relation to both the DNA concentration and the AT composition.

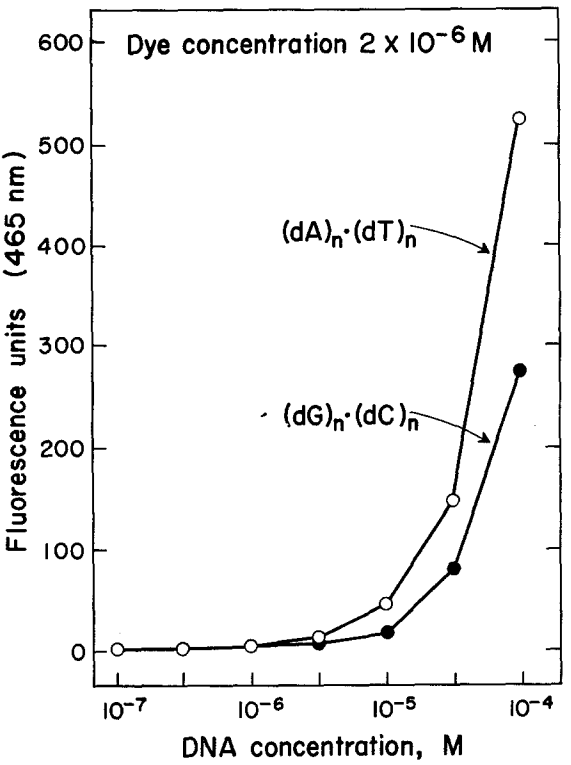

Fig. 3. Enhancement of bibenzimidazole (H 33258) fluorescence by DNA homopolymers. — The fluorescence of 2×10^{-6} *M* 33258 at 465 nm was determined in the presence of increasing concentrations of $(dA)_n \cdot (dT)_n$ and $(dG)_n \cdot (dC)_n$, as in Fig. 2. The polymers used were purchased as the duplex from P. L. Biochemicals

Finally, we attempted to assess the relative contributions of an AT and GC polymer to the observed fluorescence changes. The results are shown in Fig. 3. We note that both $(dA)_n \cdot (dT)_n$ and $(dG)_n \cdot (dC)_n$ enhance fluorescence and that the former is twice as active as the latter in this reaction. We therefore conclude that in natural DNA polymers both AT and GC base-pairs contribute to fluorescence enhancement but that the enhancement by AT is significantly greater than by GC. Thus we can rule out other models according to which GC base-pairs either do not contribute to fluorescence enhancement, or actually quench fluorescence as in the case of 9-aminoacridine or quinacrine (Weisblum, 1973a).

The relative contributions of localized variations in chromosomal DNA concentration or of "accessibility" to the observed cytofluorometric differences have not been assessed in this study; we have determined the *potential* of DNA polymers with varying base-compositions to modify fluorescence intensity. Possible contributions of localized variations in chromosomal DNA concentration or of accessibility are secondary in nature and would only serve to modify expression of the potential established above; the contribution of these factors to cytological fluorescence in individual cases remains to be evaluated.

While it is clear that polynucleotides modify bibenzimidazole fluorescence, we have not yet established whether the differential effects observed are solely proportional to binding of the dye by DNA. In order to establish this point, it will be necessary to determine the capacity of DNA to bind the dye as well as the affinity constants which characterize this reaction. Thus, a model according to which both AT and GC base-pairs enhance fluorescence of bound bibenzimidazole with equal efficiency but that the differences observed are due to different affinities, remains a distinct possibility. Regardless of the exact physical-chemical details of the mechanism of the enhanced fluorescence, our data allow us nevertheless to conclude that 33258 can be useful in distinguishing chromosome regions which are enriched for AT base-pairs, and the specificity of this compound is different from that of quinacrine in regard to the response to G.

Additional indirect support for our interpretation of bibenzimidazole fluorescence comes from studies of primate (Seth and Gropp, 1973), murine and bovine (Gropp *et al.*, 1973) chromosomes. In these studies, as well as in the previous work of Hilwig and Gropp (1972), it was noted that those chromosome regions which fluoresce brightly with quinacrine ("Q-bands") also fluoresce brightly with 33258. According to our model of quinacrine fluorescence, the Q-bands of human chromosomes are relatively rich in AT base-pairs. This assignment is consistent with the observations of Bobrow and Madan (1973) that Q-bands appear to be relatively easily denatured at 85° C in 0.07 *M* phosphate buffer, pH 6.8, as well as with the immunofluorescence studies of Schreck *et al.* (1973) in which anti-adenosine and anti-cytidine antibodies were employed as fluorescent stains of human metaphase chromosome preparations.

From a combined knowledge of the base composition of mouse centric DNA and the fluorometric properties of 33258 in the presence of a series of DNAs of known base composition, we have been able to propose an interpretation which is consistent with the available experimental data. These studies suggest that the specificity of 33258 involves fluorescence enhancement by AT-rich DNA in a manner somewhat different from quinacrine. This compound will no doubt be useful for

cytogenetic studies of other organisms whose chromosomes contain adjoining regions with differing average base compositions, as well as for identifying AT-rich chromosome regions which show reduced fluorescence intensity in quinacrine-treated preparations.

Acknowledgments. This work was supported by grants IN-35-15 from the American Cancer Society, GB-17108-1 from the National Science Foundation, and GM-20871-01 from the National Institutes of Health.

We thank Dr. H. Loewe (Hoechst GmbH, Frankfurt am Main) for providing the sample of Hoechst 33258 used in these studies, and Dr. P. K. Seth for informing us of his cytofluorometric studies prior to publication. R. Demars, K. Patau, and R. D. Wells made many helpful suggestions.

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Received March 25, 1974 / Accepted by H. Bauer
Ready for press April 18, 1974

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