Reverse Banding on Chromosomes Produced by a Guanosine-Cytosine Specific DNA Binding Antibiotic: Olivomycin

Abstract. *Characteristic reverse fluorescent banding patterns (R bands) on human, bovine, and mouse metaphase chromosomes are produced by treating chromosome preparations directly with olivomycin. With the DNA in solution, the repeating polymer poly[d(G-C)] · poly[d(G-C)] (where G is guanine and C is cytosine) enhanced the fluorescence of olivomycin, while the antibiotic fluorescence was not affected by the alternating polynucleotide poly[d(A-T)] · poly[d(A-T)] (where A is adenine and T is thymine). Calf thymus DNA, with an intermediate G-C content of about 40 percent, showed a smaller fluorescence enhancement in the presence of olivomycin as was observed for the synthetic polynucleotide poly[d(G-C)] · poly [d(G-C)]. The closely related antibiotic chromomycin A_3 showed the same results as were obtained with olivomycin either in the solution interaction with specific DNA's or with the metaphase chromosome preparations. The production of R bands by these G-C–specific DNA binding antibiotics lends credence to the suggestion that the arrangement of the nucleotide sequences along the chromosome is a primary determinant for the appearance of fluorescent bands.*

The discovery that certain DNA-binding fluorochromes produce specific banding patterns on chromosomes (*1*) led to investigations searching for other DNA-binding agents having such specificity (*2, 3*). Fluorescent labels found to date show a great affinity for sequences rich in A-T (adenine-thymine) base pairs and result in the production of Q bands (*3–5*). If the base composition around the intercalation or binding site of the fluorochrome is to be the primary determinant for the appearance of bands (*3, 4, 6*), it should be possible to find compounds which will bind specifically to G-C (guanine-cytosine) base pairs, show enhanced fluorescent intensity in the presence of DNA with high G-C content, and also produce a reverse fluorescent banding pattern on chromosomes (R bands). Here we report the discovery of a group of DNA-binding agents which exhibit those characteristics: the chromomycin-like antibiotics olivomycin, chromomycin A_3, and mithramycin (*7*).

Olivomycin, chromomycin A_3, and mithramycin are chemically similar compounds; they contain a central chromophore to which five different carbohydrate moieties are attached (see the structure of olivomycin in Fig. 1). These closely related antibiotics have essentially identical mechanisms of action; all form stable complexes with DNA in vitro (*8*). Direct interaction of these drugs with DNA was shown by cosedimentation with DNA (*9*), and by a shift to longer wavelength in the absorption spectrum of the antibiotic on addition of DNA in the presence of divalent cations, such as Mg^{2+} (*10, 11*). The chromomycin-like antibiotics require native DNA for complex formation (*12, 13*). In addition, the number of binding sites of these antibiotics rises with increasing G-C content of the DNA. The mode of binding of this class of antibiotics to DNA has not been described in molecular terms, although the small conformational changes observed upon binding of these antibiotics to DNA negates the intercalation model of interaction (*14*).

The fluorescence intensity of olivomycin and chromomycin A_3 in the presence of several DNA's was measured on a Turner 430 spectrofluorometer. Olivomycin, in the presence of Mg^{2+}, exhibits an excitation maximum at 405 nm and an emission maximum at 525 nm. In the presence of DNA, small spectral shifts are observed for both the absorption (maximum at 440 nm) and emission spectra (maximum at 532 nm). Figure 2 shows that olivomycin's fluorescence is not affected by the repeating polymer poly[d(A-T)] · poly[d(A-T)]. A similar result was obtained for the interaction of chromomycin A_3 with this polymer. This finding is in agreement with the lack of binding of chromomycin to this polymer (*13*) and the absence of inhibition of transcription of poly[d(A-T)] · poly[d(A-T)] in the presence of this antibiotic (*11*). In contrast, a strong enhancement of the olivomycin (or chromomycin A_3) fluorescence is observed in the presence of the alternating polynucleotide poly[d(G-C)] · poly[d(G-C)]. This result is comparable to the 100 percent inhibition of transcription of poly(dG) · poly(dC) observed in the presence of chromomycin A_3 (*13*). Calf thymus DNA, with an intermediate G-C content (42 percent) showed a fluorescence enhancement in the presence of olivomycin (or chromomycin A_3), but the increase in fluorescence was less than that observed in the presence of poly[d(G-C)] · poly[d(G-C)]. This finding agrees with the lesser number of binding sites of calf thymus DNA for chromomycin A_3 as compared to poly[d(G-C)] · poly[d(G-C)] (*13*).

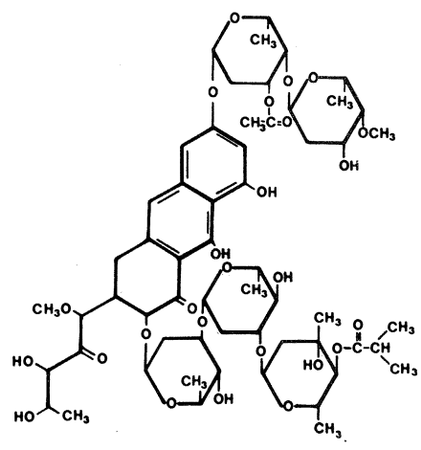


Fig. 1. The structure of olivomycin.

Characteristic fluorescence patterns of metaphase chromosomes treated with olivomycin were observed. The banding patterns on chromosomes were produced by directly staining the slides with the antibiotics. Routinely air-dried chromosome preparations from short-term cultures of human and bovine lymphocytes were used. A well-defined yellow fluorescent banding pattern along the human chromosomes appeared to be similar to both the reverse bandings (R bands) obtained by the R staining method (*15*), and the "reversed" banding pattern produced by acridine orange (*16*). The bright fluorescent bands produced by olivomycin correspond to the dark staining bands obtained from the reverse-staining Giemsa method or the bright yellow bands obtained from the reverse fluorescent banding technique in which acridine orange is used. These two R banding techniques require prior treatment of the chromosome preparation in a hot phosphate buffer (approximately 86°C) before staining is carried out. The R bands on human chromosomes treated with olivomycin are obtained by simply treating the slide of the chromosome preparation in an olivomycin solution for 20 minutes at room temperature. The staining solution consisted of 1 mg of olivomycin per milliliter in phosphate buffer at a *p*H of 6.8. The slide was then washed in two changes of buffer solution for a total of 2 minutes and mounted with the same buffer (*p*H = 6.8). Preparations were examined with a Zeiss WL fluorescent microscope equipped with a dark-field condenser for transmitted illumination. The light source was a d-c powered HBO W/2 mercury burner. A BG-12 excitation filter was used and the barrier filter was set at No. 50.

Characteristic R banding patterns on the human chromosome were clearly observed. These bands faded quickly under

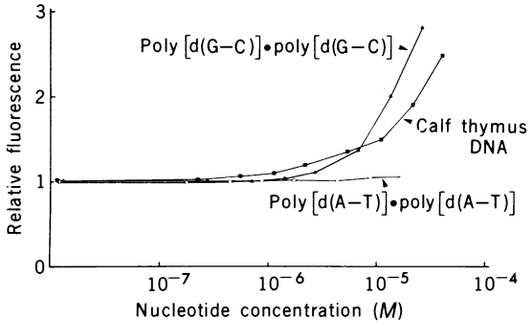


Fig. 2. Relative fluorescence of olivomycin in the presence of varying concentrations of DNA. Excitation wavelength is 440 nm; emissions wavelength, 532 nm. The olivomycin concentration was $5 \times 10^{-6}M$, and the DNA concentrations are expressed in nucleotide phosphorus. Experiments were carried out in $0.01M$ $NaPO_4$ (*p*H 6.8), $0.001M$ $MgCl_2$, and 0.1 mM EDTA.

the fluorescent microscope, possibly through the photodecomposition of the antibiotic. However, each individual chromosome can be identified by the R banding pattern along the chromosomes (Fig. 3). The distal long arm of the Y chromosome showed negative fluorescence. In addition, certain chromosomal regions, for example, the short arm of chromosome 22, showed variable bright fluorescent intensity between the homologs in every cell examined (Fig. 3). The same variant chromosomes were also identified by the acridine orange R banding technique. Thus, the banding patterns along the chromosomes and the variable fluorescent regions on specific chromosomes of man revealed by the olivomycin were in general the same as obtained by the acridine orange R banding procedure. This similarity was also confirmed by using the bovine chromosome preparation. All centromeric heterochromatin regions of the chromosomes with the exception of the X chromosomes in the cow demonstrated bright fluorescence with the olivomycin banding procedure (Fig. 4). A similar finding was reported previously when the conventional acridine orange R banding technique was used (*17*).

In addition, it has been shown that the mouse centromeric regions contain repetitive DNA that is rich in A-T base pairs (*18*) and these regions would be expected to stain negatively with a G-C–specific fluorochrome. It was observed, indeed, that mouse chromosome preparations subjected to the olivomycin procedure showed nonfluorescent centromeric regions.

R bands were also observed on chromosome preparations treated with mithramycin and chromomycin A_3. However, the fading of the bands was so rapid that to record the bands by microscopic photography was almost impossible.

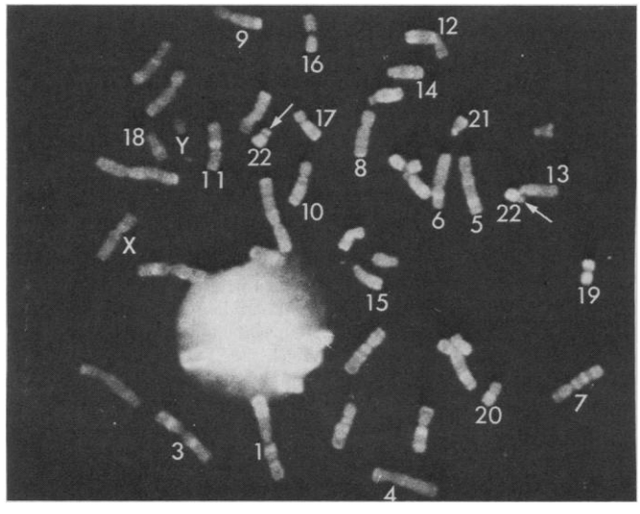
The simplicity of the R banding procedure with olivomycin and its elimination of the necessity to treat (and risk destroying) the chromosome preparations before staining them makes this procedure a useful tool for chromosome analysis, especially if the problem of fading of the bands can be somehow overcome. Other excitation sources, such as a xenon lamp or laser, might reduce fading problems with these specific fluorochromes.

Several aspects of the use of the chromomycin-like antibiotics in producing R bands are of interest:

1) The selective labeling of chromosome DNA with olivomycin and other chromomycin-like antibiotics clearly differentiates specific fluorescent patterns on different regions of human and bovine metaphase chromosomes. The characteristic reverse fluorescent bands produced by these compounds introduce a new dimension not only in analysis and identification of chromosomes but also in searching for further understanding of the structure and function of these chromosome regions.

2) Unlike the interaction of other antibiotics, such as quinacrine, with DNA, the interaction of the chromomycin-like antibiotics with DNA is not affected by high ionic strength. Flourescence fading of the bands is still a limitation in the use of olivomycin, but different excitation procedures may overcome this.

3) This group of antibiotics might provide some insight into the mechanism of chromosome banding. The fluorescence enhancement of olivomycin (or chro-



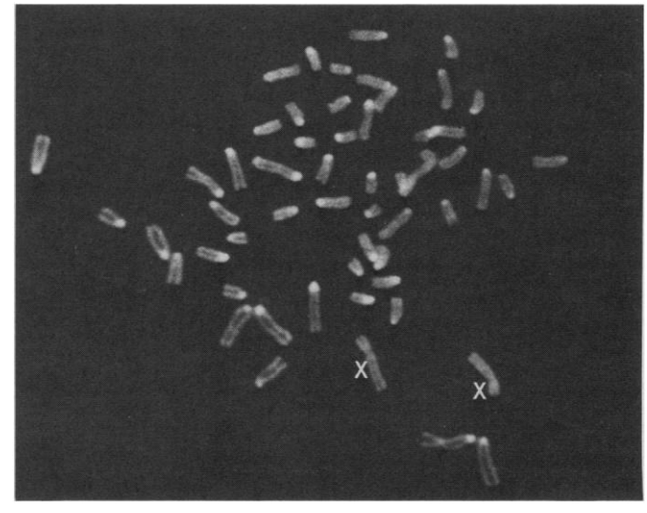


Fig. 3 (left). A metaphase spread of a human lymphocyte from a male subject. The characteristic R banding pattern on chromosomes was produced by staining the slide of the chromosome preparation with olivomycin solution in a concentration of 1 mg/ml. (Olivomycin was dissolved in Sorrenson's buffer, a mixture of $0.07M$ $NaPHO_4$ and $0.07M$ KH_2PO_4 with a *p*H of 6.8). One homologous chromosome from each pair of autosomes and the sex chromosomes were labeled by their designated numbers and by X and Y, respectively. The short arm of a chromosome 22 was brighter than the short arm of its homologs (indicated by arrows). The distal long arm of the Y chromosome appears to be fluorescent negative. The exposure time for the negative of the photograph was 2 seconds with Kodak Tri-X Pan film (ASA = 400; ×40 planapochromat objective and ×10 eyepiece). Fig. 4 (right). A metaphase spread of a lymphocyte from a cow. Distinct bright fluorescence appeared in the centromeric region of every autosome in the genome. The centromeric regions of the X chromosomes appear to be fluorescent negative. The staining technique and microscopic photography are as described in Fig. 3.

momycin A_3) in the presence of natural DNA is best rationalized as an expression of the degree of binding of these antibiotics. The degree of binding is a reflection of the G+C percentage of the DNA under observation. This mechanism is different from the mode of binding of aminoacridines (quinacrine) to DNA, where the binding to DNA is not sequence specific but the variable quantum efficiency of fluorescence is a function of the base composition at the binding site. The production of R bands by the G-C–specific fluorochromes supports the suggestion that nucleotide sequence arrangement along the chromosome plays an important role in determining the display of fluorescent bands (*3, 4, 6*).

Further qualitative examination of chromosome banding with these and other fluorescent probes should provide additional information on the mechanism of chromosome banding, and also may lead to a better understanding of the structure and function of chromosomes.

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Hyperphenylalanemia: Effect on Brain Polyribosomes Can Be Partially Reversed by Other Amino Acids

Abstract. *The effect of a single injection of phenylalanine (2 mg/g of body weight) on brain polyribosomes, which increases the number of inactive monoribosomes, persists for 2 to 3 hours. A single injection of seven large neutral amino acids after phenylalanine administration results in a reversal of the effect on brain polyribosomes with a resultant decrease in monoribosomes to near normal levels. The other common amino acids are apparently not limiting during hyperphenylalanemia, because an injection of these did not increase recovery.*

A single injection of phenylalanine leads to a shift in polyribosome profiles of mammalian brain tissue and increases the number of monoribosomes (*1–3*). The monoribosomes are apparently inactive and accumulate as a result of a decreased rate of the initiation of protein synthesis (*3*). In addition to the effects on protein metabolism, a state of hyperphenylalanemia produces a rapid and extensive decrease in the intracellular concentration of several amino acids in brain cells both in vivo and in vitro (*4*). The amino acids which are most affected are the large neutral amino acids which are cotransported with phenylalanine. Alterations in the intracellular concentrations of these amino acids have been suggested as the mechanism by which phenylalanine decreases protein synthesis in neural tissue (*4, 5*). If this amino acid imbalance is directly responsible for the inhibition of neural protein synthesis, then the administration of the proper amino acids to hyperphenylalanemic animals might restore the normal balance and reverse the inhibitory effect on neural protein synthesis.

The injection of neonatal mice with L-phenylalanine (2 mg per gram of body

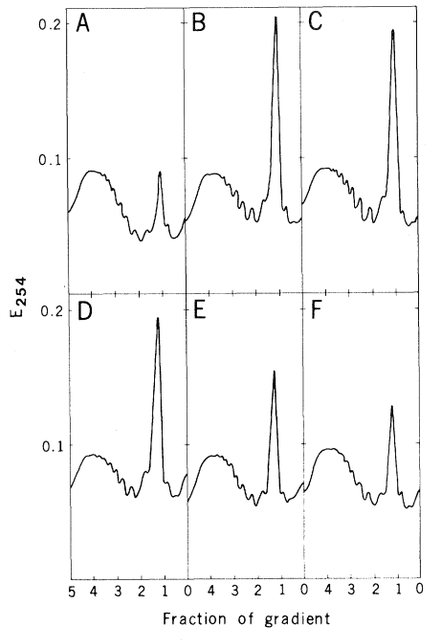

Fig. 1. Sedimentation profiles of brain polyribosomes isolated after injection of various amino acids. Noninbred Swiss albino mice (*6*) received intraperitoneal injections of phenylalanine as described (*3*) at a dose of 2 mg/g of body weight (65 μl/g) from a stock solution containing 30 mg of L-phenylalanine per milliliter of saline; control animals received a comparable amount of saline alone. (A) Saline was injected 60 minutes before the animals were killed (the same profile is found if saline is injected 90 to 180 minutes before killing). (B) Phenylalanine was injected 60 minutes before the animals were killed. (C) Phenylalanine was injected 120 minutes before the animals were killed (similar polyribosome profiles were observed for at least 180 minutes after injection with phenylalanine). When two injections were used, the second injection was given 60 minutes after the administration of phenylalanine. The second injection consisted of a mixture of the seven large neutral amino acids (*7*) (3.75 mg of each amino acid per milliliter of saline, injected at a dose of 0.075 mg of each amino acid per gram of body weight, or 20 μl/g). (D) Phenylalanine was injected 60 minutes before a saline injection (20 μl/g); animals were killed either 30 or 60 minutes later. (E) Phenylalanine was injected 60 minutes before the seven amino acids were injected; the animals were killed 30 minutes later. (F) Phenylalanine was injected 60 minutes before the seven amino acids were injected; animals were killed 60 minutes later. Brain polyribosomes were isolated as described (*3*) by homogenizing brains with a motor-driven glass-Teflon homogenizer in TKM buffer solution (50 m*M* tris-HCl, *p*H 7.2; 25 m*M* KCl; 5 m*M* $MgCl_2$) containing 1 mg/ml of bentonite. The resulting homogenate was centrifuged at 8400*g* for 15 minutes at 4°C. The supernatant fluid containing the polyribosomes was removed and sodium deoxycholate and Tween-40 were added to a final concentration of each of 1 percent. The polyribosomes were then purified by gel filtration through a Sephadex G-200 column (1.1 cm by 10 cm) at 4°C. The brain polyribosome preparations were then layered (approximately 2.0 E_{254} units) on a 5.0-ml linear (0.7 to 1.5*M*) sucrose gradient in TKM buffer and centrifuged at 190,000*g* for 105 minutes in a Beckman SW 50.1 rotor at 4°C. Duplicate gradients were run for each condition in each experiment. After centrifugation the sucrose was removed from the top of the gradient by a peristaltic pump, the optical absorption at 254 nm (E_{254}) was continuously monitored with an ISCO model UA-2 ultraviolet analyzer, and the area under the monoribosome and polyribosome region was determined (*3*).