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DISTAMYCIN INHIBITS THE BINDING OF A NUCLEAR FACTOR TO THE -278/-256 UPSTREAM SEQUENCE OF THE HUMAN HLA-DRα GENE

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Abstract—In this study we analyse the effects of the anti-tumor compound distamycin on the binding of nuclear factor(s) to a synthetic oligonucleotide (GTATA/IFN-γ) mimicking a putative regulatory region of the human HLA-DRα gene. This region contains the sequence (GTATA), that is required for nuclear protein binding and is likely to interact with distamycin. The present results, by showing that distamycin inhibits the interaction between nuclear factors and the GTATA/IFN-γ oligonucleotide, suggest that distamycin might alter the binding of transacting factors to *cis*-elements containing AT/TA sequences. Alterations of nuclear protein binding to specific target sequences could be one of the molecular mechanism(s) by which distamycin exerts its antiproliferative activity on living cells.

Distamycin [1, 2] is an antibiotic exhibiting strong antiviral as well as antitumor activity [3]. Although the biochemical mechanism of action of distamycin and related compounds is not completely understood, recent data suggest that this drug is capable of forming reversible complexes by making contact points in the minor groove of AT-rich DNA sequences [4–8].

Crystallographic studies of DNA complexes with distamycin have demonstrated the importance of H-bonds involving the ligand NH groups of this drug and adenine N-3 as well as thymidine O-2 on double stranded DNA [6]. These conclusions have been supported by NMR studies [9].

AT-rich sequences are often found in eukaryotic promoters in regions crucial for their biological activity [10]. Besides the TATA box, additional boxes containing TAT or ATA motifs are found in 5′ regions of eukaryotic genes [10]. These 3-tuples are not randomly distributed throughout the genomic sequences of eukaryotic genes, exhibiting low frequency in the coding portions and higher frequency in the 5′ regions [10]. This peculiar distribution may have been generated during evolution to allow selective binding of transcriptional factors to the N-TAT-N and N-ATA-N sequences present in many regions involved in transcriptional regulation and binding to nuclear factors [10]. For instance, the nuclear factor SRF/f-EBP binds to the sequence GATGTCCATATTAGGACATC of the human c-*fos* oncogene [11, 12]; the Eryf-1/NFE-1 factor binds to the sequence GATAAG of the γ-globin genes [13]; the nuclear B factor binds to the sequence motif TATAAGTA of the histone H3 of *Drosophila* [14]. A compilation of transcription regulatory proteins and their corresponding target DNA sequences can be found elsewhere [15].

Since distamycin selectively binds AT/TA containing DNA sequences, one might hypothesize that this drug interferes with the binding of nuclear factors to AT-rich target DNA sequences. We have recently found that a sequence at position -278 from the start of transcription of the human HLA-DRα gene (GTATA) is important for the binding of nuclear factors [16]. In this study we analyse the effects of distamycin on these sequence specific interactions.

MATERIALS AND METHODS

Cell lines

The human B-lymphoid WI-L2 [17], the melanoma MNT-1 [17] and MRN-1 [16], and the promyelocytic HL-60 [18] cell lines used in this study were grown in α-medium (GIBCO) supplemented with 10% FCS in 5% CO_2 at 37°.

Chemicals

Synthetic oligonucleotides. The nucleotide sequences of wild-type synthetic oligonucleotides [GTATA/IFN-γ (44 bp), IFN-γ (23 bp) and z (45 bp)] and the mutant M-GTATA/IFN-γ synthetic oligonucleotide are shown in Fig. 1B. 5′-3′ Strands and the relative complementary 3′-5′ strands were synthesized on a Pharmacia Gene Assembler Plus DNA Synthesizer using the phosphoramidite method. Equimolar amounts of each strand were 5′ labelled with [γ-^{32}P]ATP by the enzyme T4 polynucleotide kinase (Genenco), combined, heated for 5 min at 80° in 0.5 M NaCl and annealed for at least 30 min at room temperature (RT) [19].

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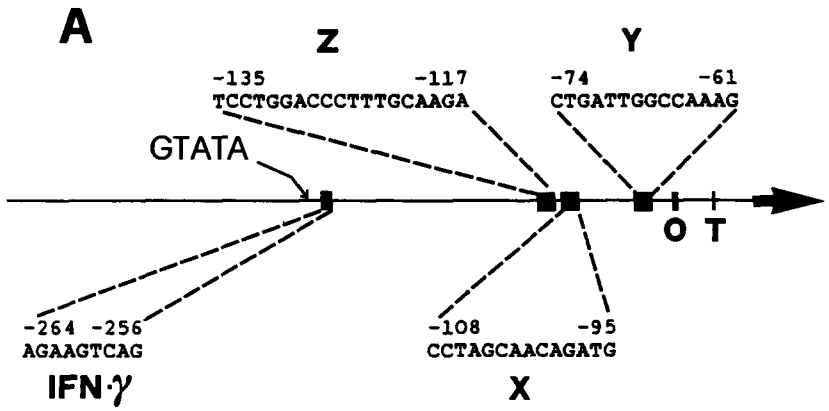

B

GTATA/IFN-γ mer	5'-CTAATGTGCTTCAGGTATATCCCTGTCTAGAAGTCAGATTGGGG-3' 3'-GATTACACGAAGACCATATAGGGACAGATCTTCAGTCTAACCCC-5'
M-GTATA/IFN-γ mer	5'-CTAATGTGCTTCAGAGCCCTCCCTGTCTAGAAGTCAGATTGGGG-3' 3'-GATTACACGAAGTCTCGGGAGGGACAGATCTTCAGTCTAACCCC-5'
IFN-γ mer	5'-TGTCTAGAAGTCAGATTGGGG-3' 3'-ACTGATCTTCAGTCTAACCCC-5'
Z mer	5'-ATCTTGTGTCCTGGACCCTTTGCAAGAACCCTTCC-3' 3'-TAGAACACAGGACCTGGGAAACGTTCTTGGGAAGG-5'

Fig. 1. (A) Promoter region of the human HLA-DRα gene showing the location of the GTATA, IFN-γ, z, x and y sequences. O = octamer; T = TATA box. (B) Synthetic oligonucleotides used in the present study. The GTATA and IFN-γ sequences are underlined.

Distamycin. Distamycin (synonyms distamycin A, stallimycin) hydrochloride was obtained by total synthesis at the laboratories of Menarini Ricerche Sud, Pomezia (Rome, Italy) [1].

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (shortly, gel retardation) was performed as originally described [20] with minor modifications. Nuclear extracts were prepared according to Dignam *et al.* [21] at a protein concentration (BCA assay, Pierce, Rockford, IL, U.S.A.) of 1–5 mg/mL. Binding reactions were, unless otherwise specified, set up in binding buffer (20 mM Tris–HCl, pH 7.6, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA,* 0.01% Triton X-100, 5% glycerol, 0.5 mM spermidine), in the presence of increasing amounts of poly d(I-C).poly d(I-C) (Pharmacia, Uppsala, Sweden), 1 μg of nuclear extract proteins and 0.25 ng of end-labelled double stranded oligonucleotides (approximately 50,000 Cherenkow counted cpm), in a total volume of 25 μL. After 30 min at room temperature, samples were electrophoresed at constant voltage (300 V for 2 hr) through a low ionic strength (0.35 × TBE buffer) (1 × TBE = 0.089 M Tris–borate; 0.089 M boric acid; 0.008 M EDTA) on 10% polyacrylamide gels until tracking dye (Bromophenol blue) reached the end of a 16 cm slab.

Gels were dried and exposed at −80° with intensifying screens. The order of addition of the reagents was the following: (a) poly d(I-C).poly d(I-C); (b) competitor DNA (if required); (c) distamycin; (d) labelled oligonucleotides; and (e) nuclear extracts [20].

RESULTS

Binding of nuclear factors to the -278/-256 region of the human HLA-DRα gene

The map of the 5′ region of the human HLA-DRα gene [22] is shown in Fig. 1A. The GTATA oligonucleotide identifies a region required for nuclear protein binding [16]. Synthetic oligonucleotides employed in this study are indicated in Fig. 1B. Mutated M-GTATA/IFN-γ mer contains the sequence AGCCC instead of GTATA, IFN-γ lacks the GTATA box.

Figure 2 shows the binding to the GTATA/IFN-γ oligonucleotide of nuclear factors from different human cell lines. Most of the retarded bands (for

* Abbreviations: mer, oligonucleotide; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; HLA, histocompatibility antigens; IFN-γ, γ-interferon.

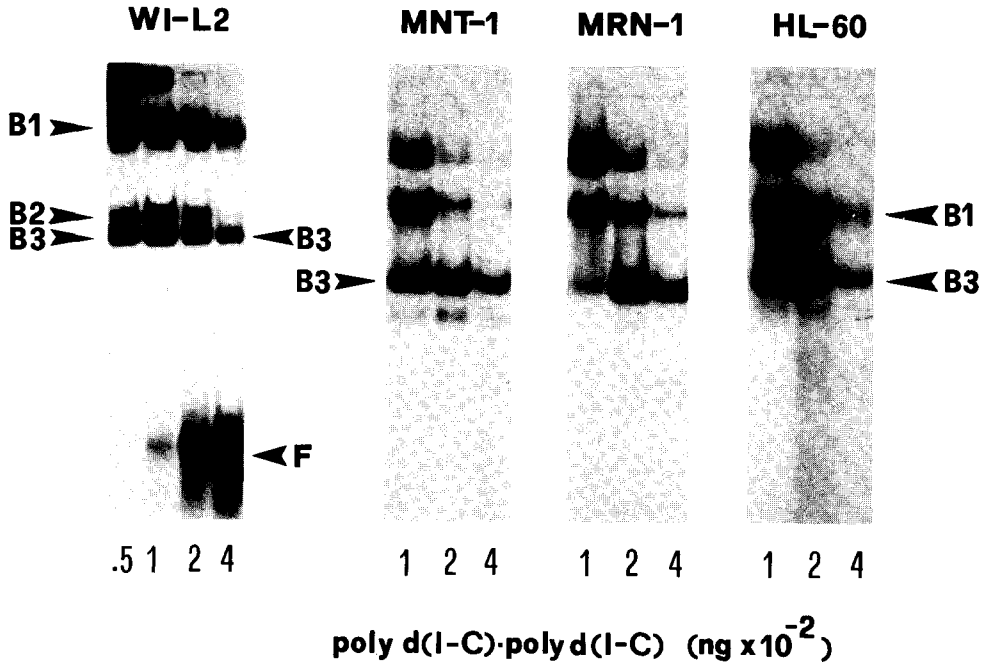

Fig. 2. Gel retardation assays of the binding of nuclear proteins from different cell lines to the GTATA/IFN-γ oligonucleotide. One microgram of nuclear extracts from the B-lymphoid WI-L2, the melanoma MNT-1 and MNR-1, and the promyelocytic HL-60 cell lines were allowed to bind to 0.25 ng of 5′ end-labelled GTATA/IFN-γ mer in a final volume of 25 μL in the presence of the indicated amounts of poly d(I-C).poly d(I-C). After 30′ binding at room temperature the reaction mixtures were electrophoresed on 8% polyacrylamide gels. B1–B3 indicate retarded bands; F = free GTATA/IFN-γ 5′ end labelled mer.

instance B1 and B2) tend to disappear when high concentrations of poly d(I-C).poly d(I-C) are present in the reaction mixture, whereas the B3 retarded band is clearly detectable even at the highest concentrations (400 ng/reaction) of the non-specific competitor. The specificity of the B3 band is further sustained by the evidence that it cannot be displaced by excess amounts of oligonucleotides mimicking the z sequence, and is inhibited in a dose dependent manner by the cold GTATA/IFN-γ mer (Fig. 3). Taken together, these results indicate that the B3 retarded band represents nuclear factor(s) displaying high affinity for the GTATA/IFN-γ oligonucleotide.

Mutations of the GTATA sequence alter the binding of the -278/-256 oligonucleotide to the B3 factor

Figure 4 provides evidence that alterations of the GTATA sequence affect the binding of the B3 factor(s). In fact, no B3 band is detected when the 23-mer oligonucleotide (IFN-γ), lacking the GTATA box, is used in gel retardation experiments (Fig. 4A). In addition, no B3 band is detected (Fig. 4B) using an oligonucleotide (M-GTATA/IFN-γ) modified by five base transversions in correspondence to the GTATA box (Fig. 1B). Accordingly, displacement of the B3 factor(s) from the GTATA/IFN-γ mer was achieved with cold GTATA/IFN-γ but not with cold M-GTATA/IFN-γ mer (data not shown).

These results suggest that the GTATA sequence is required for nuclear protein binding to the -278/-256 upstream region of the HLA-DRα gene.

Inhibitory effects of distamycin on the binding of the B3 factor to the GTATA/IFN-γ oligonucleotide

Figure 5A shows that distamycin inhibits the binding of the B3 nuclear factor(s) to the GTATA/IFN-γ oligonucleotide.

In this experiment 25–200 μM (final concentration) distamycin was added to approximately 0.25 ng of ^{32}P-labelled GTATA/IFN-γ oligonucleotide and 400 ng poly d(I-C).poly d(I-C). After 5 min, binding buffer and nuclear extracts were added and the reaction mixture was further incubated at room temperature for 30 min. A dose dependent inhibition of the binding of the B3 factor(s) was detected. The lack of increase of free GTATA/IFN-γ oligonucleotide is probably due to the fact that the other non-specific retarded bands (as for instance the B1 retarded band) are not affected by the distamycin treatment. In addition, we have evidence showing that distamycin does not cause breaking of the oligonucleotides employed in this (Fig. 5B) and other studies (Gambari *et al.*, unpublished results).

Fifty per cent inhibition in the binding of the GTATA/IFN-γ oligonucleotide to the B3 factor(s) was consistently observed when 50 μM of distamycin were used in the gel retardation incubation mixture.

DISCUSSION

In this report we present two complementary sets of experiments. First, we provide evidence that the

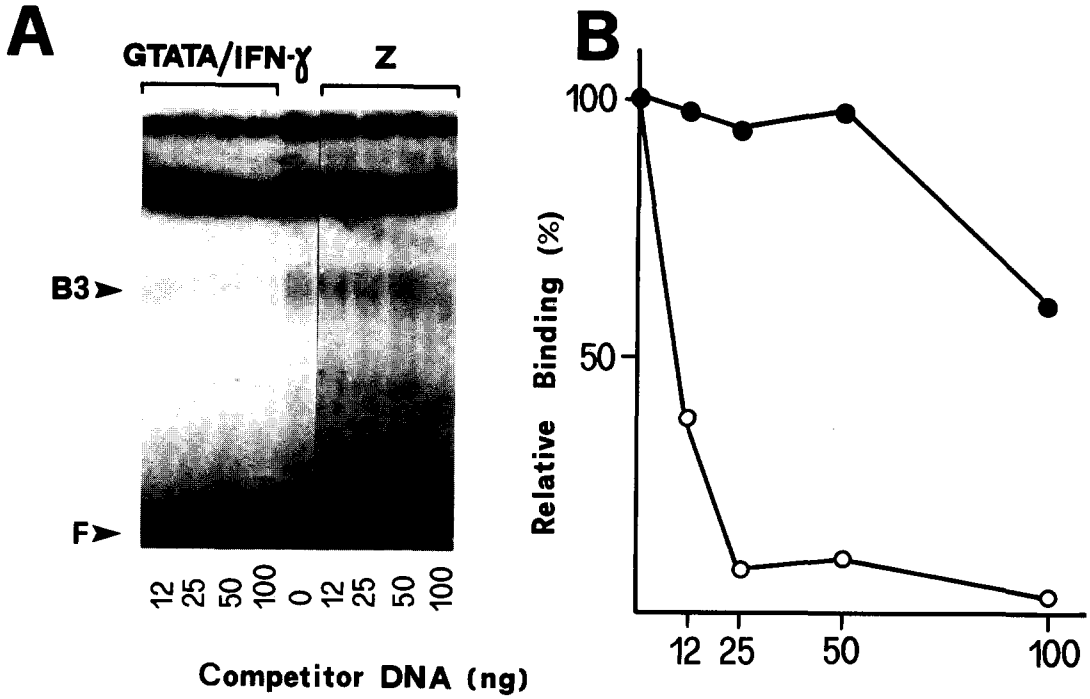

Fig. 3. Competition by the GTATA/IFN-γ (○) and the z box (●) mers in the binding of the ^{32}P-labelled GTATA/IFN-γ mer to the B3 factor(s). (A) Autoradiograms; (B) densitometric analysis. In this experiment the nuclear extracts were from the MRN-1 cell line. The gel retardation experiment was carried out as indicated in Fig. 2, in the presence of 200 ng of poly d(I-C).poly d(I-C) and the indicated concentrations of unlabelled GTATA/IFN-γ and z box oligonucleotides.

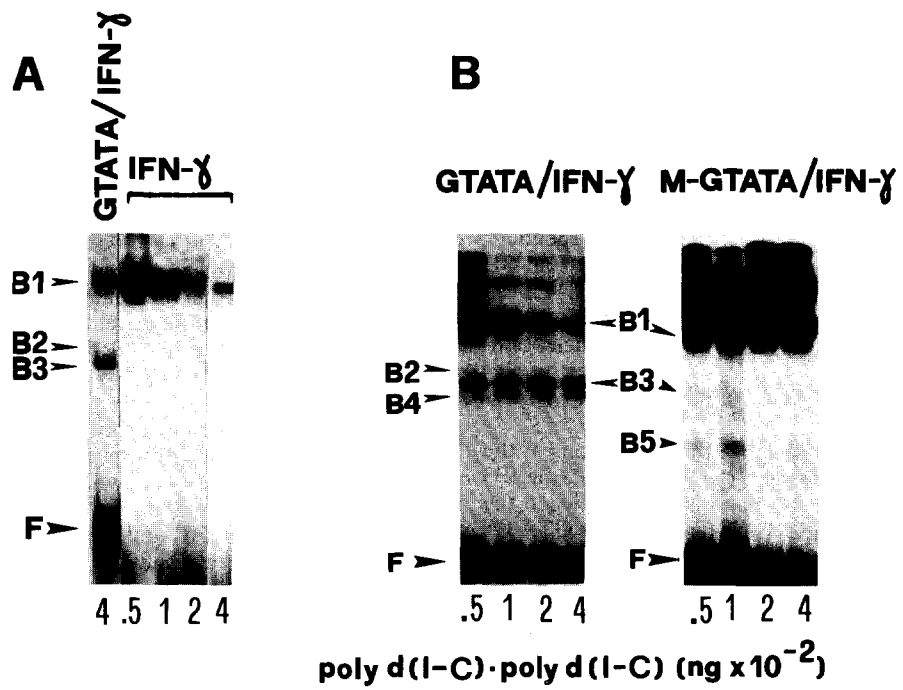

Fig. 4. (A) Binding of nuclear factor(s) to the IFN-γ mer, lacking the GTATA box. (B) Differential binding of the B3 factor(s) to the GTATA/IFN-γ and the mutated M-GTATA/IFN-γ mers. In this experiment nuclear extracts from the MRN-1 cell line were used. The amounts of poly d(I-C).poly d(I-C) competitor are indicated. Binding reactions (25 μL) were carried as described in Fig. 2. The sequences of the oligonucleotides employed are shown in Fig. 1B.

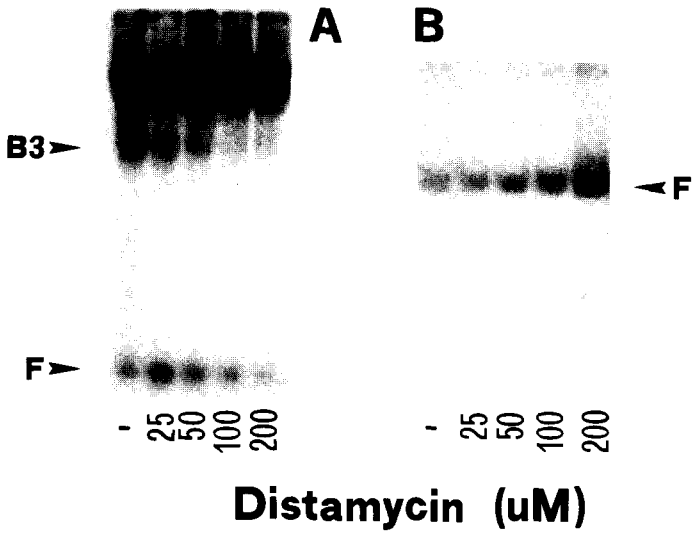

Fig. 5. Effects of distamycin on the binding of the nuclear factor B3 to the GTATA-IFN-γ mer (A) and on the stability of the GTATA-IFN-γ mer (B). Increasing amounts of distamycin were added to ^{32}P-labelled GTATA/IFN-γ mer and 400 ng of poly d(I-C).poly d(I-C). After 5 min, binding buffer was added with (A) or without (B) nuclear extracts, the reaction was carried on for 30 min at room temperature, and electrophoresed on 8% polyacrylamide gels. B3 indicates the specific binding between nuclear factor(s) and the GTATA/IFN-γ mer; F = free GTATA/IFN-γ mer.

GTATA sequence, located in the -278/-256 region of the HLA-DRα promoter [16], is specifically recognized by nuclear protein(s).

Although the presence of several low affinity complexes was detected (such as those identified by the B1 and the B2 retarded bands), one complex seems GTATA specific on the basis of (a) its presence in all the cell lines tested; (b) resistance to displacement by non-specific competitor DNA; (c) peculiar mobility; (d) lack of binding to mutant oligonucleotides; and (e) lack of displacement by mutant oligonucleotides.

Second, we show that distamycin, which displays strong binding specificity to AT/TA rich DNA sequences [5–9], is in fact able to alter the activity of such sequence specific DNA-binding proteins. Therefore, the binding of nuclear factor(s) to the GTATA/IFN-γ mer exemplifies an experimental model system to screen drugs and other compounds interfering with sequence specific protein–DNA recognition processes.

GTATA is a "rare" (under-represented) oligosequence not randomly distributed within the promoters of mammalian genes [10, 23], most often located around the −40 (TATA box) [24] and −270 regions in several genes [16]. Therefore, the antibiotic distamycin might be the prototype of drugs capable of modulating both *in vitro* and *in vivo* the expression of large batteries of genes sharing ATA/TAT rich regulatory sequences.

Inhibition in the binding of nuclear proteins to target gene sequences by distamycin has so far been observed only in a few eukaryotic genes. Broggini *et al.* [25] recently reported that distamycin inhibits the binding of OTF-1 and NFE-1 from K562 cells to their target sequences, ATGCAAAT and GATAAG, respectively. In agreement with these data, also in our study AT/TA dinucleotides are present in the sequences involved in DNA–protein binding and distamycin displacement [16].

In conclusion, the present work suggests that drugs which specifically bind to different, though simple, DNA sequences could be of interest in exploring the mechanism of activation of genes displaying a CG/GC rich promoter (such as the Ha-ras-1 oncogene) [15] versus genes displaying a AT/TA rich promoter. Whether the mechanism of action of distamycin on living cells is related to the *in vitro* phenomena described in this paper remains to be proven.

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