Antibodies directed against guanylic acid and guanosine are nucleolus specific probes

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Received 29 July 1985

Purified antibodies against guanylic acid and guanosine binding to RNA at guanosine residues were used to probe human lymphocyte preparations by indirect immunofluorescence. Neither antibody gave any banding pattern with metaphase chromosomes but both showed binding to specific sites in the interphase nuclei. Evidence presented indicates that these sites are guanosine residues on rDNA transcripts at the nucleolar organizer regions.

Indirect immunofluorescence *Guanylic acid antibody* *Human lymphocyte* *Guanosine binding*

1. INTRODUCTION

The eucaryotic chromatin contains mainly DNA, 5 types of histones and non-histone proteins organized in a manner which allows the complex processes of replication, transcription and modification to take place in an ordered way. To study the organization of chromosomes, different types of banding methods have been used. Definite correlations have been made as to what the bands represent in terms of underlying DNA structure [1–3].

Antibodies to histone and non-histone proteins have been successfully used to study many structural and functional aspects of chromatin [4–7]. Antibodies against adenosine, thymidine and cytosine have been used to study chromatin by indirect immunofluorescence and have lent support to the earlier finding that Q- and G-bands represent regions of AT-rich and R-bands the regions of GC-rich sequences in DNA [8]. We have recently purified high affinity antibodies specific to pG which bind to RNA specifically at guanylic acid residues but not to DNA [9] and also high affinity antibodies specific to guanosine which bind to RNA and also to ssDNA specifically at guanosine residues [10]. Human lymphocyte preparations were examined by indirect immunofluorescence using the above mentioned antibodies. It was found that antibodies bind to specific localized regions in interphase nuclei but do not give a banding pattern with chromosomes. The antibody binding sites in interphase nuclei are identified as guanosine residues on rDNA transcripts at nucleolar organizer regions.

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Abbreviations: dsDNA, double-stranded DNA; EDC, 1-ethyl-3-(3-dimethylisopropyl)carbodiimide; FITC, fluorescein-isothiocyanate; G^{ox-red}, guanosine oxidized with $NaIO_4$ and reduced with $NaBH_4$ (also for other nucleosides); G-bands, Geimsa bands; IgG, immunoglobulin-G; pG, guanylic acid; Q-bands, quinacrine bands; R-bands, reverse bands; ssDNA, single-stranded DNA; γ-G, γ-globulins

2. MATERIALS AND METHODS

Nucleotides, nucleosides, FITC and silver nitrate were obtained from Sigma (St. Louis, MO).

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Antibodies against guanylic acid and guanosine were elicited in rabbits [9,10] using thyroglobulin-pG (prepared by the EDC method) and BSA-guanosine (prepared by the periodate method) conjugates, respectively, as immunogens. Specificities of pG antibodies were studied by their binding to [^{3}H]pG, [^{3}H]RNA and [^{32}P]DNA and their competition by non-radioactive ligands using nitrocellulose filter assay [9]. Antibodies against guanosine were similarly probed with [^{3}H]G^{ox-red}, [^{3}H]RNA and [^{32}P]DNA [10]. The antibodies were purified by affinity chromatography. Antibodies were highly specific to the corresponding haptens. pG specific antibodies bind to [^{3}H]RNA at guanylic acid residues but do not bind to dsDNA or ssDNA [9]. Guanosine specific antibodies bind both to [^{3}H]RNA and [^{32}P]ssDNA at guanosine residues [10].

2.1. *FITC-conjugation of anti-rabbit IgG*

The procedure of Clark [11] was used with some modifications. FITC (2 mg) was added to γ-globulin preparation of anti-rabbit IgG-antisera raised in goat (25 mg/ml in carbonate buffer, pH 9.5) and stirred. The extent of reaction was monitored in small aliquots of the reaction mixture by the A_{280}/A_{495} of the excluded fraction on a column of Sephadex G-50. Reaction was stopped when the A_{280}/A_{495} was 1. After removing the unreacted FITC by dialysis, the material was loaded on a DEAE-cellulose column and the bound material was eluted batchwise with increasing concentrations of NaCl. The material with A_{280}/A_{495} eluted with 0.5 M NaCl. The yield was 7.5 mg/ml of antisera.

2.2. *Culturing of human lymphocytes*

Metaphase preparations were made from leucocytes cultured from whole blood according to Hungerford [12] as modified by Krishnamurthy et al. [13].

2.3. *Binding of antibodies and their visualization*

The slides containing the lymphocyte preparations were denatured in 95% formamide for 60 min. Then pG or guanosine antibodies were added followed by FITC-conjugated anti-rabbit IgG. The slides were washed, mounted and observed under fluorescence microscope-Zeiss Photomicroscope-III, using a blue interference filter set 455–490, FT-510 chromatic splitter and LP-520 orange filter. Plain slides were observed under phase contrast. Kodak plus-X-400 ASA and ORWO-NP-27 films were used. Photographs were taken at a magnification of 64 ×, 1.5 NA oil immersion.

2.4. *Silver-staining*

The procedure of Goodpasteur and Bloom [14] as modified by Krishnamurthy (unpublished) was used.

3. RESULTS

Fig.1A shows the fluorescence photomicrograph of the binding of purified pG antibodies to human lymphocyte preparations. There is no banding pattern with metaphase chromosomes. Certain localized fluorescence spots can be observed in interphase nuclei. Lymphocytes were cultured thrice. From each culture at least 5 slides (5 × 2 cm) each were prepared with the antibody and with normal rabbit γ-globulins. Each of the slides was scanned completely under the fluorescence microscope and the number of cells showing the localized fluorescence in the slides containing the antibody was almost 100% and those in the slides containing normal γ-globulins was $<0.01\%$. The same was true with 10 μg of purified guanosine antibodies. The results were the same with either 80 μg of anti-pG or anti-guanosine γ-globulins but for the higher background fluorescence. This localized fluorescence in interphase nuclei was seen in almost all interphase nuclei in the preparations. The control slides where the same amount of normal rabbit IgG was used instead of antibodies did not show this localized fluorescence (fig.1B).

As already mentioned pG antibodies bind to RNA but not to ssDNA and guanosine antibodies bind both to RNA and ssDNA. Since similar patterns are produced by both guanosine and pG antibodies, RNA can be expected to be taking part in the binding to interphase nuclei. To confirm this, the slides containing the lymphocyte preparations were treated with RNase (0.12 mg/ml) for 1, 2 and 3 h. The slides were scanned and scored as above but with 2 lymphocyte preparations. The fluorescence intensity was considerably reduced after 1 h, was barely detectable after 2 h and totally lost after 3 h of treatment, showing that the

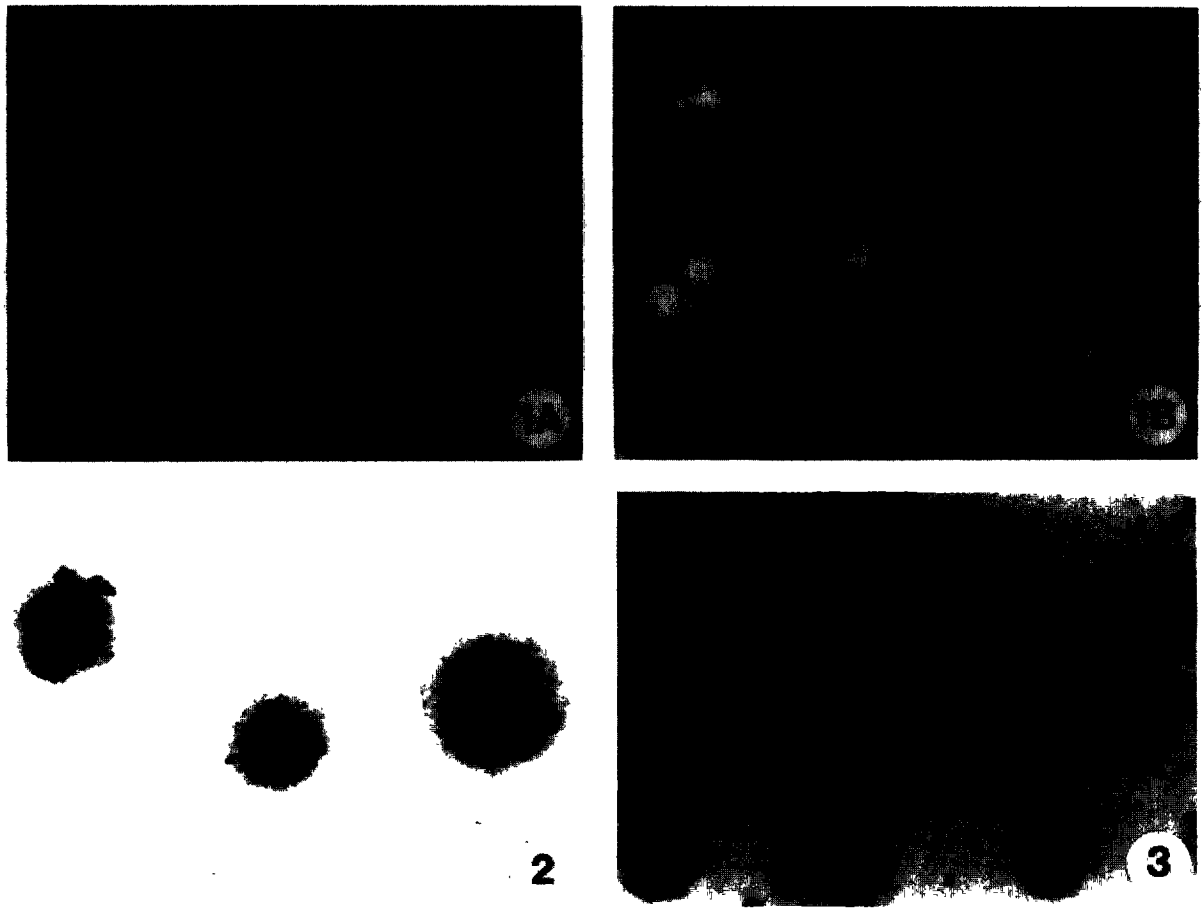

Fig.1. (A) Guanosine and guanylic acid antibodies binding to lymphocyte preparations. Slides containing the lymphocyte preparations were treated with 95% formamide in SSC (0.15 M NaCl + 0.015 M trisodium citrate) at 65°C for 60 min, then washed in PBS (0.02 M phosphate buffer, pH 7.5, + 0.59% NaCl) for 5 min. 25 μl (10 μg) of purified pG antibodies were applied and covered with a cover glass, incubated at room temperature for 3 min then washed in PBS for 5 min. 25 μl (50 μg) of FITC coupled anti-rabbit IgG were then applied, covered with cover glass and left at room temperature for 30 min, again washed in PBS for 5 min, mounted in McIlvaine's buffer (0.02 M phosphate buffer, pH 7.5 + glycerol = 1:1 v/v) and observed under the fluorescence microscope. Two control experiments were done: 1, normal rabbit γ-G were used instead of antibodies, and 2, denaturation treatment was omitted. (B) Normal rabbit IgG binding to lymphocyte preparations. Lymphocyte preparations were treated as described for (A) but normal rabbit-IgG was used instead of pG or guanosine antibodies.

Fig.2. Silver staining of interphase nuclei. Slides were covered with 1 g/ml solution of silver nitrate and incubated at 65–70°C for 5 h in a moist chamber. Then slides were washed thoroughly in distilled water and stained with Leishman's stain (0.15% in methanol) for 5 min and observed under microscope.

Fig.3. Silver staining after formamide denaturation. Slides were treated as in fig.2 after denaturation in 95% formamide in SSC (0.15 M NaCl + 0.015 M trisodium citrate) at 65°C for 60 min.

localized fluorescent spots are produced by the binding of guanosine and pG antibodies to RNA.

The specificity of binding was further probed by competition experiments with nucleosides. Only guanosine and G^{ox-red} compete for binding at low concentrations and other nucleosides do not have any effect on the fluorescence pattern even at very high concentrations (table 1). This clearly shows

Table 1

Specificities of guanosine antibody binding to interphase nuclei[a]

Inhibitor	Fluorescence intensity at different concentrations of the competitor (nM)			
	1	10	100	1000
Guanosine	+	0		
G^{ox-red}	+	0		
Adenosine	+ + +	+ + +	+ + +	NT
Inosine	+ + +	+ + +	+ + +	NT
Uridine	+ + +	+ + +	+ + +	NT
Cytidine	+ + +	+ + +	+ + +	NT
A^{ox-red}	+ + +	+ + +	+ + +	+ + +
U^{ox-red}	+ + +	+ + +	+ + +	+ + +

[a] Experiment was as in fig.1 except that the corresponding amount of competitors (in 25 μl) were also added to the slides with the antibody preparations

One slide (2.5 × 2.0 cm) was scanned for each compound. The indicated scoring was observed in at least >90% of the cells. + + +, fluorescence intensity same as that without competitor; +, faint fluorescence detectable only under high magnification (400 ×); 0, fluorescence intensity same as that with normal rabbit γ-G; NT, not tested

that the antibodies are binding at guanosine residues. It was thought highly probable that the guanosine and pG antibodies bind to rDNA transcripts at the nucleolar organizer regions to produce the fluorescence pattern observed because rRNA genes are localized in a cytologically recognizable organelle, the nucleolus, and they exhibit dosage repetition [15] and are transcriptionally highly active in the interphase when the demand for RNA is high.

Silver-staining is a standard method to specifically visualize the extent of transcriptional activity at nucleolar organizers [16,17]. To confirm that the antibodies are binding at nucleolar organizer regions, the silver-staining pattern in interphase nuclei was compared with that of guanosine- and pG-antibody binding (figs 1A and 2). Both patterns are very similar. Silver grains are distributed at 2–10 sites in interphase nuclei. The extent of binding to guanosine and pG antibodies also varies in different interphase nuclei. It is known that silver binds to a protein associated with rDNA transcripts [18,19]. Lack of any silver staining in formamide treated slides (fig.3) lends further support to the fact that silver may be binding to a protein associated with rDNA transcripts. Guanosine and pG antibodies bind to guanosine residues on RNA in interphase nuclei only after denaturation with formamide.

4. DISCUSSION

Antibodies to guanosine have been used earlier to study human metaphase chromosomes [20,21]. It was observed that they do not give any banding pattern with formamide denatured chromosomes, as confirmed here. Indirect immunofluorescence studies reported here show that guanosine and pG antibodies bind at specific sites in interphase nuclei (fig.1A). These sites were suspected to be RNA as pG antibodies have been shown not to bind to DNA. This was confirmed by the effect of RNase treatment on the fluorescence pattern. Competition experiments further confirmed the guanosine-specificity of the binding (table 1). A suitable denaturation treatment is usually given to lymphocyte preparations to enable anti-nucleoside/tide antibodies to bind to chromosomes, as these antibodies cannot bind to dsDNA. Here the formamide treatment is probably serving the purpose of denaturing or removing the protein covering the RNA transcripts or denaturing the RNA transcript itself.

Similarity of pattern obtained by silver-staining and antibody binding show that the antibodies bind to rDNA transcripts at the nucleolar organizer regions. Busch and coworkers have examined the nucleolar immunofluorescence produced by the binding of nucleolus-specific antibodies [19,22–27]. The fluorescence pattern reported by them is similar to the pattern we have obtained with guanosine and pG antibodies. Recently, a monoclonal antibody specific for human nucleolar protein has been reported [28] and the fluorescence pattern shown by those antibodies (28) is very similar to that observed here using guanosine and pG antibodies. Photomicrographs of localization of RNA polymerase-I in interphase cells reported by Sheer and Rose [29] show a similar pattern to that we have obtained with guanosine and pG antibodies.

The present studies indicate that the guanosine and pG antibodies produced can be used as probes

for nucleolar organizer regions. This is significant because to understand the arrangement of chromatin in interphase nuclei, it is necessary on the one hand to obtain points of reference in the nucleus and on the other hand genetic and molecular probes for localization of genes. They are useful because of their potential role in studying the biosynthesis and processing of nucleolar RNA. In addition, since there are reports of existence of nucleolar antigens specific to human tumor cells [26,27], these antinucleolar antibodies may have diagnostic value as tumor markers. Whether rDNA transcripts can be quantitated by cytofluorimetry of the antibody binding sites is being examined.

ACKNOWLEDGEMENT

We gratefully acknowledge the receipt of microscope facilities and fruitful discussions with Dr H. Sharat Chandra.

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