J. King Saud Univ., Vol. 5, *Science* (1), pp. 11–21 (A.H. 1413/1993)

Distribution of DNA Template Activity in Normal and Carcinoma *in situ* of Human Urothelium

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(Received 14 May 1990; accepted for publication 12 April 1992)

Abstract. Acridine orange (AO) was shown to be an effective probe for the ultrastructural localization of DNA template activity in normal and carcinoma *in situ* (CIS) of human urothelium. Treatment with Rnase prior to incubation with AO did not result in a noticeable loss of AO chromatin interaction products suggesting that RNA is negligible as a target for AO.

In normal urothelium of the present study, the number of AO chromatin interaction products was found to decrease with increasing differentiation. In CIS, the DNA template activity was found to decline from basal cells to superficial cells.

Introduction

Urothelial tumors even when low grade have a high recurrence rate [1]. This could be the result of incomplete removal of the primary lesion either adjacent to or at a distance from the tumor [2]. The cytopathological characteristics of normal appearing urothelium peripheral to grossly visible tumors have been known for many years. Following the initial observation of Melicow [3], a number of investigators have pointed out the existence of cystoscopically occult precancerous urothelial abnormalities such as atypical hyperplasia and or dysplasia and nonpapillary carcinoma *in situ* in areas of the bladder not involved in obvious tumor [4, 5, 6 and 7]. The cystoscopic appearance of CIS has been described as consisting of a reddening of the mucosa with or without a change in the surface texture to give a granular oedematous, mossy or velvet appearance [8, 9 and 10].

The following account relates to biopsies collected from areas peripheral to papillary tumors and which were suspected to be instances of CIS. It was the goal of this work to study and identify differences between CIS and the normal tissue.

Materials and Methods

Tissue samples

Ten normal urothelial biopsies were obtained from patients who were kept under investigation for conditions such as nocturnal enuresis and bladder incontinence.

Carcinoma *in situ* biopsies were taken from areas adjacent to grossly visible tumors. The samples were taken from ten patients before any form of treatment was initiated.

Sample processing

The method described by Frenster [11] was applied. After fixation of 5% glutaraldehyde in medium 199 at 4° C for 2 hours at pH 7.2, the specimens were washed in medium 199. Then 1 mm - blocks of fixed tissue were treated with 0.001 M AO in medium 199 at pH 7.2 in the dark for 1 hour. The samples were next washed in medium 199 and incubated in the dark at 37° C for 30 minutes in Eagle essential medium containing 1.0 mg/ml pancreatic DNase at pH 7.4. The incubated samples were then prepared for electron microscope. Thin sections were stained with 5% uranyl acetate for 30 minutes and examined at 80 kv in an AEI - BM6 electron microscope. Control samples omitting DNase did not reveal any reaction product. For RNase treatment, the fixed samples were incubated with RNase at 37°C for 50 minutes at pH 7.2 at a concentration of 1.0 mg/ml prior to treatment with AO.

Statistical analysis

Random samples comprising 3 blocks from each of the tissues to be analyzed was used to count the electron dense reaction products within each cell. Statistical comparisons between cells of the normal urothelium and carcinoma *in situ* were made using the student t-test.

Results

Human urothelial cells that were reacted with 0.001 M acridine orange after glutaraldehyde fixation and then digested with DNase displayed characteristic electron-dense reaction products which were clearly visible by electron microscopy. The reaction product was confined to the nucleus of the cell; never found within the cytoplasm, or in the intercellular spaces of such cells. Within each cell nucleus, the reaction product was confined to extended euchromatin and never found within the condensed heterochromatin or within the nucleolar region (Figs. 3, 5, 6 and 7).

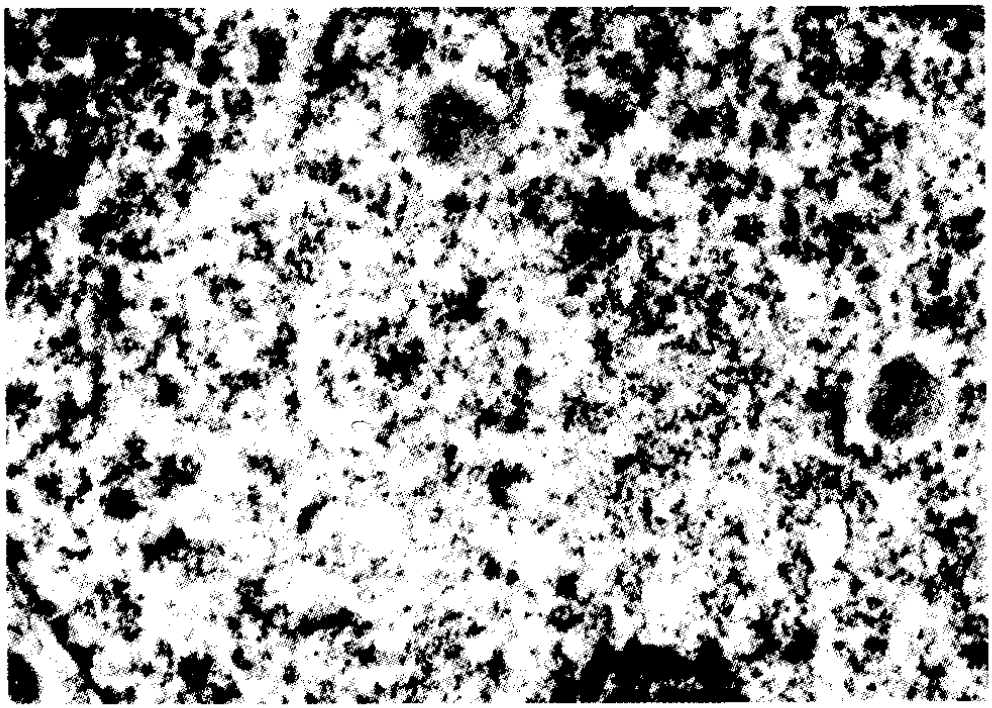

Fig. 1: Control specimen from urothelium exposed to DNase without AO treatment. The nucleus shows lack of reaction products, X 15000.

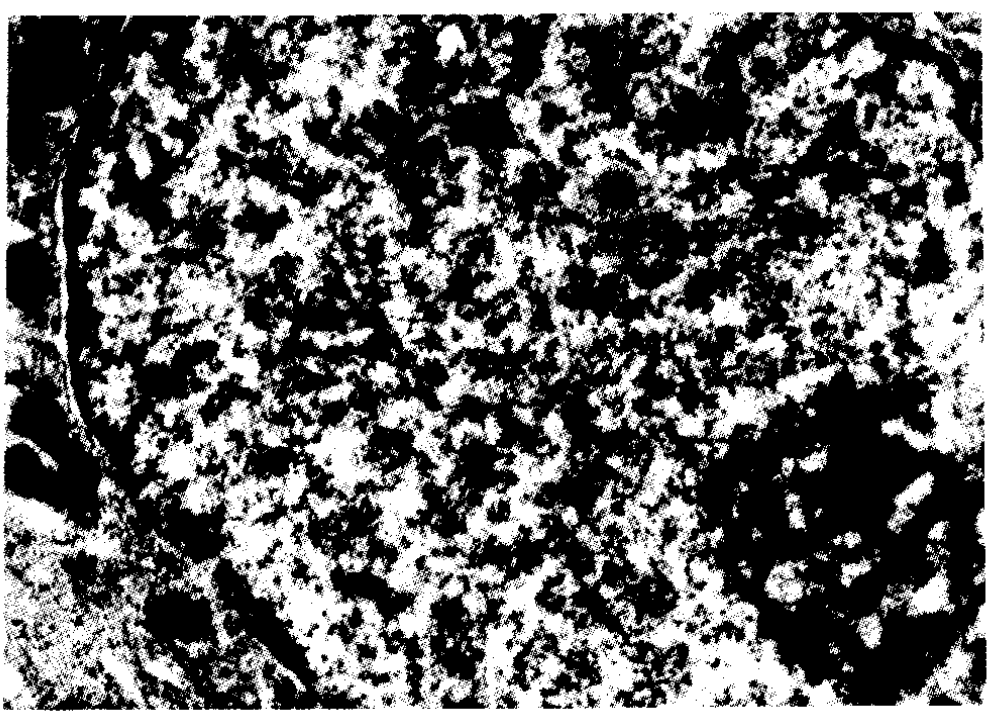

Fig. 2: Control specimen urothelium exposed to AO without enzyme treatment. The nucleus shows lack of reaction products, X 15000.

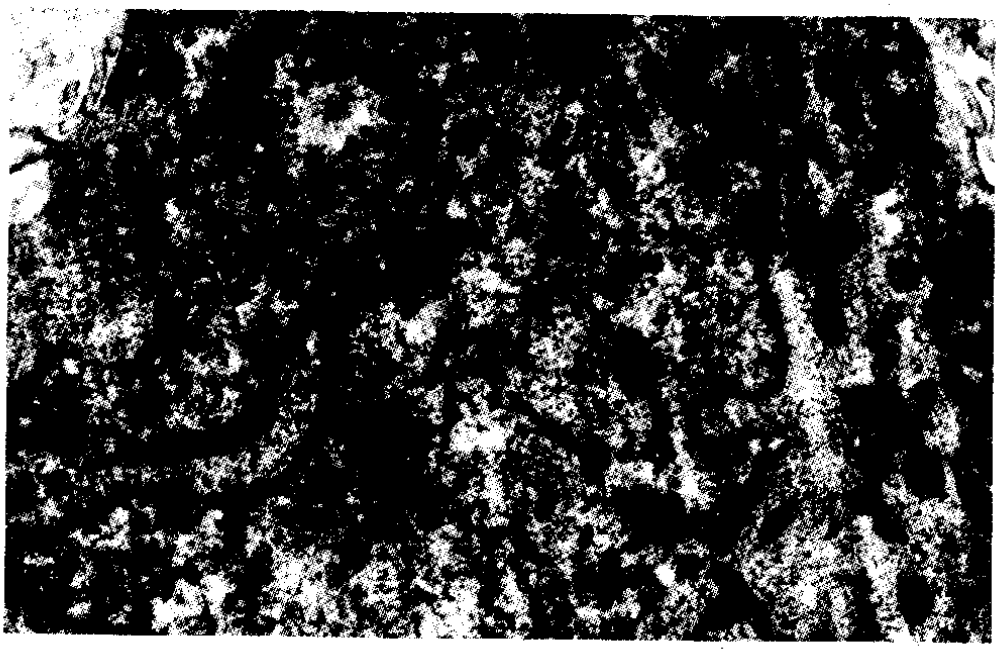

Fig. 3: Specimen from urothelium exposed to RNase prior to treatment with AO and DNase, shows the characteristic reaction products within the euchromatin portion of the nucleus (arrows), X 15000.

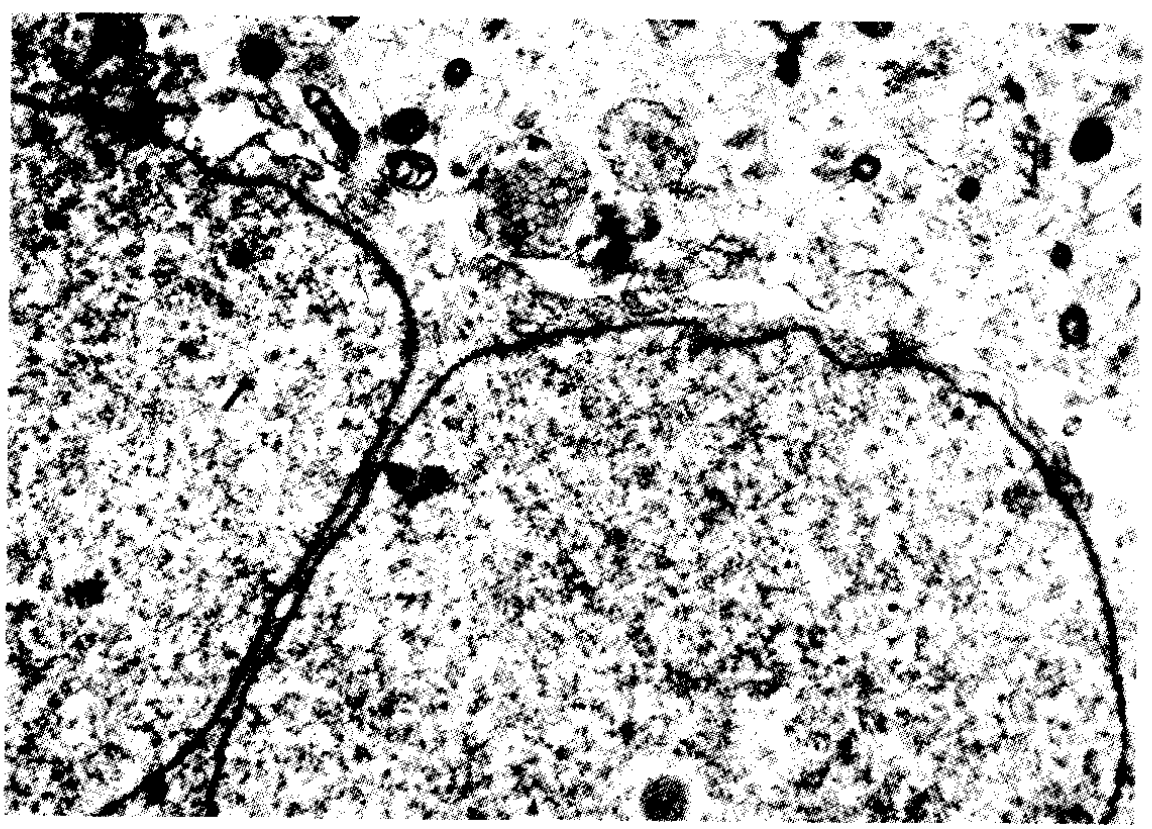

Fig. 4: Nuclei of a superficial cells of normal human urothelium. No electron-dense granules are found, X 15000.

Fig. 5: Nuclei in the intermediate and basal cells of the normal human urothelium. Electron-dense reaction (arrows) is confined to the euchromatin, X 15000.

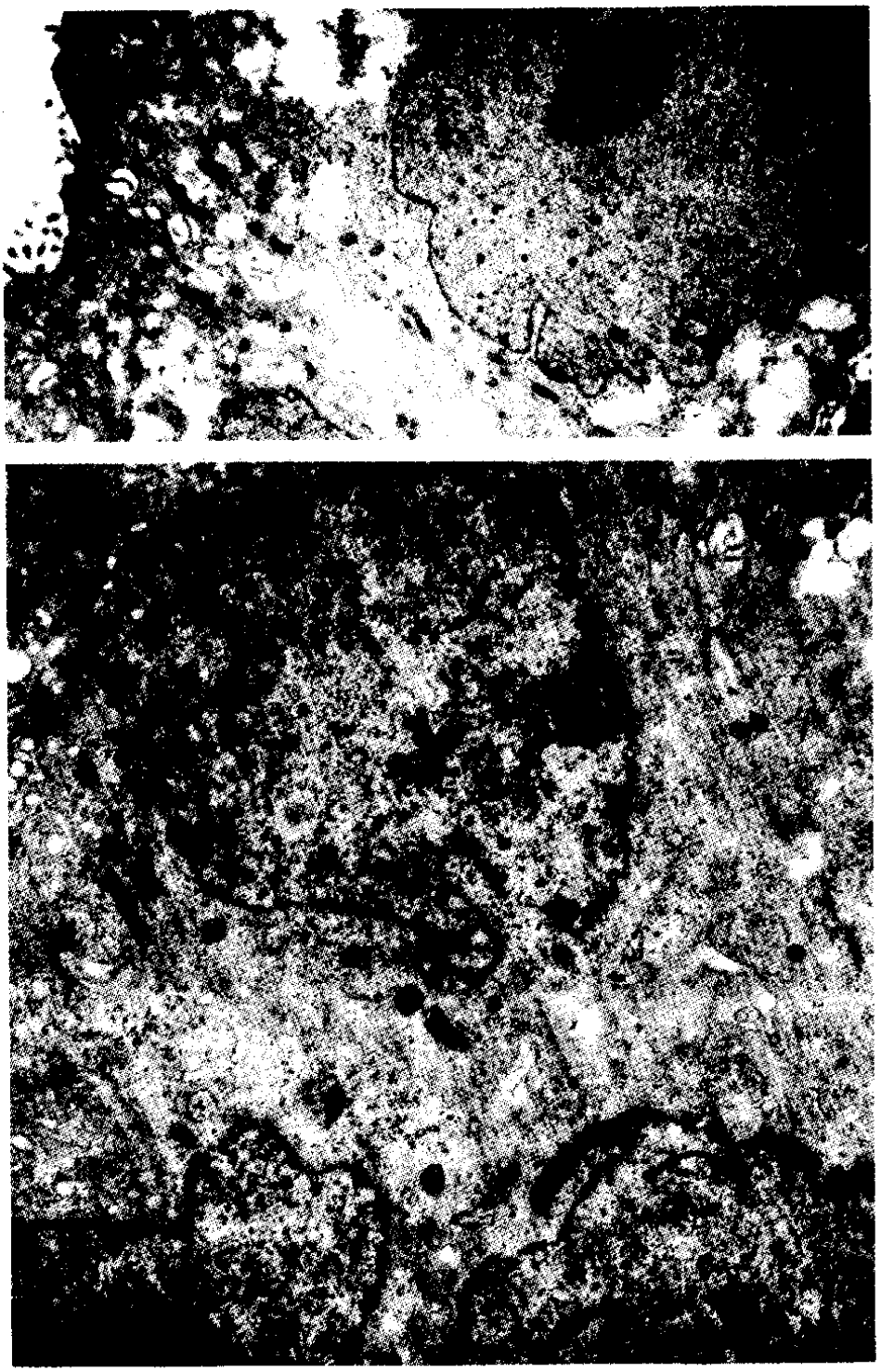

Fig. 6: Nuclei in the superficial cells of human carcinoma *in situ*. Electron-dense reaction (arrows) is confined to the euchromatin, X 15000.

Fig. 7: Nuclei in the intermediate and basal cells of human carcinoma *in situ*. Electron-dense reaction (arrows) is confined to the euchromatin, X 15000.

When AO was omitted from the preparation sequence, the characteristic reaction product was not observed (Fig. 1). Similarly when DNase was omitted from the preparation sequence the characterisric reaction product was not observed (Fig. 2). These control data indicate that both reactions with AO and digestion with DNase are necessary for visualization of the electron-dense granules.

In order to investigate whether AO interaction with chromatin might be due to RNA rather than to DNA, specimens were incubated with RNase prior to AO treatment. The incubation with RNase did not cause any substantial loss of AO chromatin products (Fig. 3) indicating that most of the electron-dense granules are the result of AO interaction with DNA.

In differentiated normal urothelium, it was found that the number of AO probes declined as differentiation progressed with the absence of any probe sites in the superficial cells (Figs. 4 and 5). Also, the number of the electron-dense granules decline but to a lesser degree in CIS. However, the number of the probes in the basal and intermediate cells was found to be significantly less than those of normal urothelium (Table I and Figs. 6 and 7).

Table 1. The mean probe count of normal and CIS of human urothlia

Cell type	Normal	CIS
Superficial	—	14.64 ± 4.23
Intermediate	53.15 ± 5.82	33.41 ± 4.85
	$P < 0.001$	
Basal	79.10 ± 5.91	40.60 ± 5.63
	$P < 0.001$	

Discussion

The current molecular model of gene derepression in mammalian chromatin indicates that histones in actively extended euchromatin are less tightly bound to the

DNA than the histones occurring in repressed, condensed heterochromatin [12]. On this basis, it might be expected that a molecular probe, such as AO which requires access to DNA to facilitate binding, would preferentially bind to DNA in euchromatin rather than to DNA in heterochromatin [13]. In the same way, DNase would interact and digest DNA in euchromatin as a co-factor in the production of the electron-dense granules [14, p 443].

The DNA serves as template for both synthesis of DNA and RNA. For a number of reasons it seems most likely that DNA templates for RNA synthesis interact with AO. Duplication of DNA as well as transcription of RNA occurs with the two strands separation. In the case of duplication, however the two strands do not completely separate before the synthesis of the new strands. Instead, synthesis of new DNA goes hand in hand with strand separation [15]. Thus AO molecules may find no space to interact with sections of the strands of DNA. Since in case of transcription only one DNA strand is transcribed, one might speculate that AO molecules might interact with sections of the corresponding DNA strand. This view is also further supported by experiments employing other biological systems. For example, in artery wall tissues stimulated by a variety of extracellular factors, the distribution patterns of 3H - thymidine labelled nuclei were completely different from those of AO positive cells [16]. However, coincidence of AO positive cells with radioactive uridine label in embryonic incisor tooth organs [14, p 446], seems to support the idea that AO specifically interact with DNA which is active as a template for RNA.

The decrease in the number of AO - chromatin interaction products per single cell nucleus during increasing differentiation of normal urothelium is in agreement with previous studies on urothelium [17 and 18, p. 227] and on other cell systems demonstrating a parallelism between decreasing cellular DNA [19, p. 565, 20 and 21].

In carcinoma *in situ* of the present study, the DNA template activity was found to decline from the basal cells to the superficial cells, but the decrease was less than that found in normal urothelium. A similar decrease in template activity has been observed in high grade papilloma of human urothelium [18, p. 227]. Also, the same kind of decrease has been observed during nuclear blebbing and maturation [22] of neoplastic Reed - Sternberg cells in the lymph nodes of untreated patients with Hodgkin's disease. It has been suggested that in the course of neoplastic cell survival a significant fraction of neoplastic cells leave the proliferative cycle by virtue of decreasing their DNA template activity [23, 24 and 25] and that might include the cells of the present material.

It can be concluded that AO is a very valuable probe for the ultrastructural localization of DNA template activity. In addition with the acquired knowledge of distribution of DNA template activities of normal and CIS in bladder urothelium the technique may have a useful application.

Acknowledgement. The authors wish to express their thanks to Prof. G.D. Chisholm and his theatre staff in the Western General Hospital, Edinburgh, U.K, for their help with the biopsies.

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توزيع نشاط قوالب الحمض الديوكسي ريبوز النووي في الخلايا الطلائية السوية وفي خلايا السرطان الموضعي لمثانة الإنسان

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(استُلم في ١٩ شوال ١٤١٠هـ؛ قُبل للنشر في ٩ شوال ١٤١٢هـ

ملخص البحث. أوضحت هذه الدراسة أن صبغ الإكردين البرتقالي فعّال في الحصر الدقيق لنشاط قوالب الحمض الديوكسي ريبوز النووي في الخلايا الطلائية السوية وفي خلايا السرطان الموضعي لمثانة الإنسان، وقد لوحظ أن المعاملة بالإنزيم المحلل للحمض الريبوز النووي قبل عملية الحضانة مع صبغ الإكردين البرتقالي لا ينتج عنها أي فقد في نواتج تفاعل الصبغ، مما يدل على أن الحمض المشار إليه ليس هدفًا للإكردين البرتقالي.

وقد أسفرت هذه الدراسة عن أن قوالب الحمض الديوكسي ريبوز النووي المسؤولة عن تصنيع الحمض الريبوز النووي تقل تدريجيًا بزيادة التميز الخلوي في الخلايا الطلائية السوية لمثانة الإنسان، وأوضحت الدراسة أن نشاط قوالب الحمض الديوكسي ريبوز النووي في خلايا السرطان الموضعي لمثانة الإنسان تتناقص تدريجيًا من الخلايا القاعدية إلى الخلايا السطحية ولكن الاختلاف في درجة النقص أقل منها في الطلائية السوية.