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Nucleolar organizer regions as a marker of incipient transformation in a model of experimental carcinogenesis

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Abstract Nucleolar organizer regions stained selectively with a silver colloid technique (AgNOR) were evaluated during the process of tumour promotion in the skin of mice. Tumour promotion and control skin samples were processed for identification of AgNOR by light microscopy and submitted to a morphometric study of the following AgNOR-related variables: nuclear area (V.NUC); AgNOR number per nucleus (N.NOR); single AgNOR area (V.NOR); total AgNOR area per nucleus (TV.NOR) and proportion of nucleus occupied by AgNOR (TV.NOR/V.NUC). N.NOR exhibited significant differences between control and tumour tissue, but in the promotion period, N.NOR did not exhibit a significant rise until week 24. V.NOR and TV.NOR rose significantly as early as 2 weeks after the onset of promotion when the cells fail to exhibit unusual microscopic features. The significant increase in AgNOR material at the beginning of the promotion period reveals the potential value of the variables assessed in the early quantitative evaluation of cellular alterations which could be linked to the probability of tumour development. Rise in AgNOR material would indicate transcriptional activation leading to an increase in protein synthesis and, ultimately, to the expression of an altered phenotype.

Key words Nucleolar Organizer Regions
Experimental carcinogenesis

Introduction

Chemical carcinogenesis is characterized by stages of cellular evolution from normal through pre-neoplastic and pre-malignant cells to highly malignant neoplasia [3, 33]. In mouse skin this is a multistage process which can be divided into at least three major components: initiation, promotion and progression [36].

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Substantial evidence supports the involvement of free radicals in the process of tumour promotion [21, 22, 24, 32] and a number of free radical-generating compounds are tumour promoters. The most studied of these compounds is benzoyl peroxide (BzPo) [37]. Although the mechanisms of promotional activity are not resolved, it is clear that tumour promoting agents have several characteristics in common including the ability to induce DNA synthesis and cell proliferation in the target cell [4].

Several reports have suggested that variations in nucleolar organizer region (AgNOR) counts may reflect the state of activation or the degree of malignancy of the lesion involved [8, 9]. Other reports suggest that AgNOR numbers are a reflection of tumour proliferation rate rather than degree of malignancy [20]. NOR represent the chromosomal location of the genes coding for 18S and 28S ribosomal RNA (RNAr), in every organism studied [19]. These genes are transcribed by RNA polymerase I and ultimately direct ribosomal formation and protein synthesis [27]. The proteins involved in RNAr synthesis associated to transcriptionally active NOR can be selectively stained by a silver colloid technique [7, 28, 31, 34] and are visualized as black intranuclear dots under the optical microscope [18]. Thus, silver staining reveals the position of transcriptionally active NOR (AgNOR) within interphase nuclei.

The present study involves morphometric analysis of unconventional AgNOR related variables and the evaluation of AgNOR numbers, which is the variable classically considered in the literature, as a monitor of the cellular modifications induced during the process of tumour promotion. We used DMBA as an initiator and BzPo as a promoter and progressor.

Materials and methods

Sencar mice were obtained originally from Dr. A.J.P. Klein Szanto and bred in our colony at the National Atomic Energy Commission.

DMBA (7,12 dimethyl benz(a)nthracene) was purchased from Sigma Chemicals Company. BzPo (Benzoyl peroxide) was purchased from Aldrich Chemical Company. All other chemicals were obtained in the purest form commercially available.

The backs of 6–8 week old female Sencar mice were shaved at least 3 days prior to experimental use. DMBA and BzPo were both applied topically on the shaved area dissolved in 0.2 ml acetone as described earlier [12]. Briefly, skin tumours in these mice were induced using a standard two-stage initiation-promotion protocol. Initiation was accomplished by a single topical application of DMBA (20 nmoles in 0.2 ml of acetone per animal). Ten days following initiation all the mice were promoted with BzPo (20 mg). There were the following experimental groups: Initiation with DMBA and promotion with 20 mg of BzPo applied twice weekly (initiated and promoted group); a no treatment group, a group initiated with DMBA and then treated with acetone alone twice weekly (initiated, non promoted) and a non-initiated, non-promoted group treated twice weekly with acetone (non-initiated, non-promoted).

At different times during the promotion period, groups of animals from each experimental group were killed by cervical dislocation and tissue samples of the treated areas and tumours when present, were routine processed for haematoxylin and eosin staining and for silver staining.

For AgNOR identification and evaluation, silver staining was performed according to Howat et al. [18]. Processing conditions were meticulously standardized to avoid known variations in the AgNOR staining method [10]. Briefly, tissue samples were fixed in formalin for 24 h and paraffin wax embedded. Sections were cut at 4 μm, dewaxed in xylene and hydrated through decreasing grades of ethanol, followed by thorough washing in deionized water for 8–10 min. The AgNOR staining solution was prepared by dissolving powdered gelatin at a concentration of 2% w/v in deionized water over a waterbath at 60–70° C. Pure formic acid was then added to a final concentration of 1%. This solution was then mixed 1:2 volumes with 50% aqueous silver nitrate solution (freshly made each time, filtered through a 0.22 μm Millipore filter and placed in the dark before use) to give the working solution. The sections were then incubated in the dark for 45 min, washed in running, deionized water for 1–15 min, dehydrated in ascending ethanol concentrations, cleared in xylene and mounted in balsam.

Quantitative relationships exist between the average dimensions of organelles and those of their profiles on sections. The aggregate of profiles on the unit area of a section is quantitatively representative of the aggregate of organelles contained in the unit volume, so that measurement obtained from sections can be used to derive structural dimension, by means of stereological methods [40]. Silver stained sections of samples of normal and promoted epithelium including tumours (papilloma, keratoacanthoma and carcinoma [12]), were photographed employing a X 100 oil immersion objective. Ten high power fields of each section were photographed at regular intervals in control and promoted epidermis with no evidence of tumour and at random in tumours. The layers considered were the basal and suprabasal strata given their significance in the induction of early cellular alterations. Microphotographs at final magnification 2000 X were used to evaluate AgNOR related variables [5] employing a semi-automatic electronic image analyser (Kontron Messgerate MOP/AM 03-Carl Zeiss) and a computer program designed ad hoc for parameter calculation. The use of microphotographs eliminates the bias due to possible variations in section thickness (the structures considered are those within the depth of focus of the objective). Moreover, it avoids possible event selection biases.

The following variables were studied; nuclear volume (V.NUC) the profile area which yields information on the biological variable nuclear volume. The number of profiles of AgNOR per nuclear profile (N.NOR) was determined, this yields information on numbers of AgNOR per nucleus. The single profile area (V.NOR) yields information on single AgNOR volume; AgNOR profile area per nucleus (TV.NOR) and volume fraction of AgNOR in nuclei (TV.NOR/V.NUC) which yields information on the proportion of nuclear volume occupied by AgNOR, were all determined.

Statistical analysis involved nested ANOVA for each of the variables to test the significance of differences between control, promoted and tumour epithelia, and of variations during the course of promotion.

The statistical model employed considers treatments, time-points within the promotion period and tumour types as fixed factors and, within them microphotographs of skin samples are nested within mice. Mean values and variances were determined according to the Bliss semi-weight method [2] to identify the treatments responsible for the statistically significant differences found.

Presentation of results as frequency histograms subjected to the statistical analysis described allows for the dispersion of values to contribute discriminative information rather than merely enlarge the standard deviation of the mean.

Results

The histological analysis of skin from animals treated only with DMBA (initiated non-promoted control) revealed no morphological alterations when compared with skin from non-initiated animals (non-initiated, non-promoted). Twice weekly topical applications of BzPo

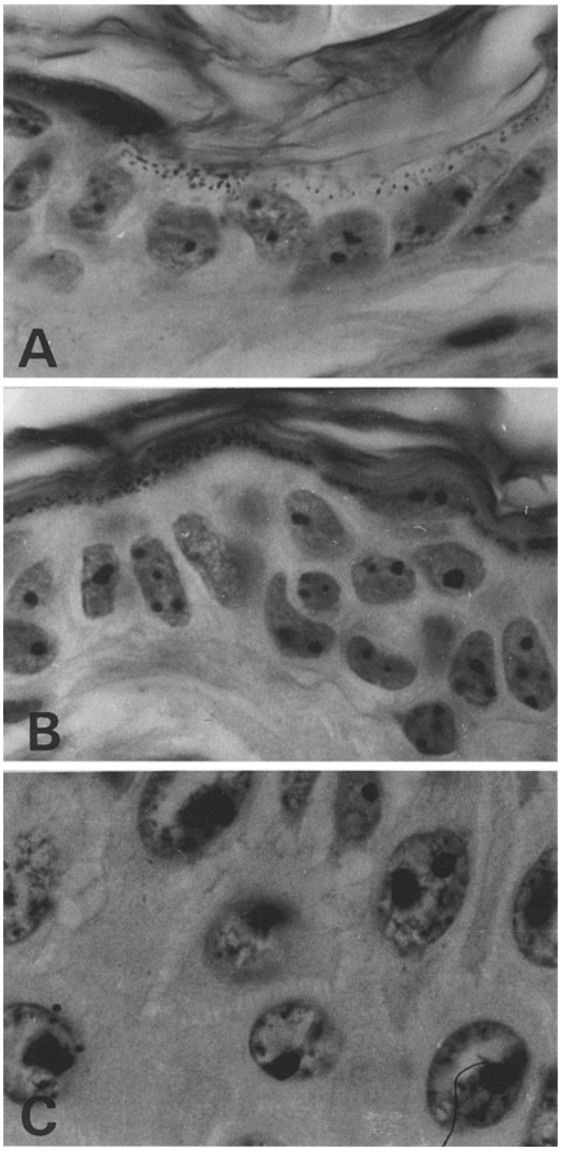

Fig. 1 Microphotograph of high-power field of representative paraffin section silver-stained to demonstrate AgNORs in control epithelium (*A*), treated epithelium at 10 weeks of promotion (*B*) and papilloma as an example of tumour tissue (*C*). ×2000

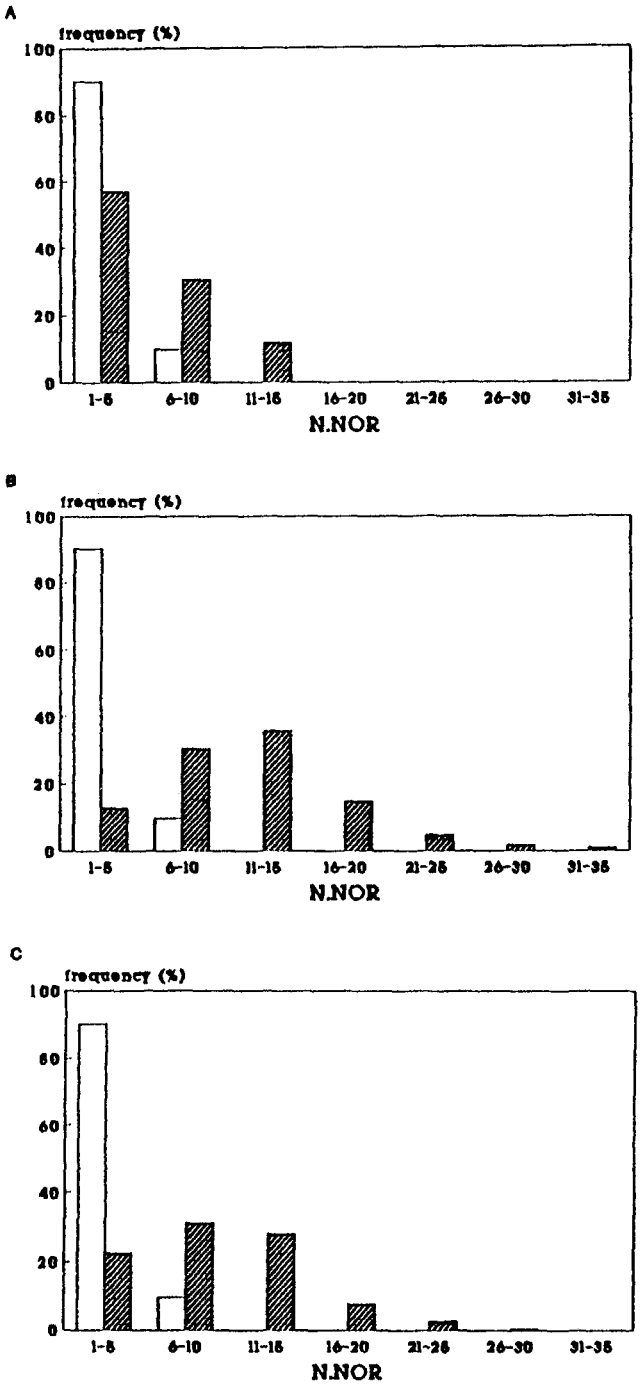

Fig. 2 Frequency distribution of number of AgNOR per nucleus (N NOR) for control (□) and tumour tissue (▨): (*A*) keratoacanthoma, (*B*) papilloma, (*C*) squamous cell carcinoma

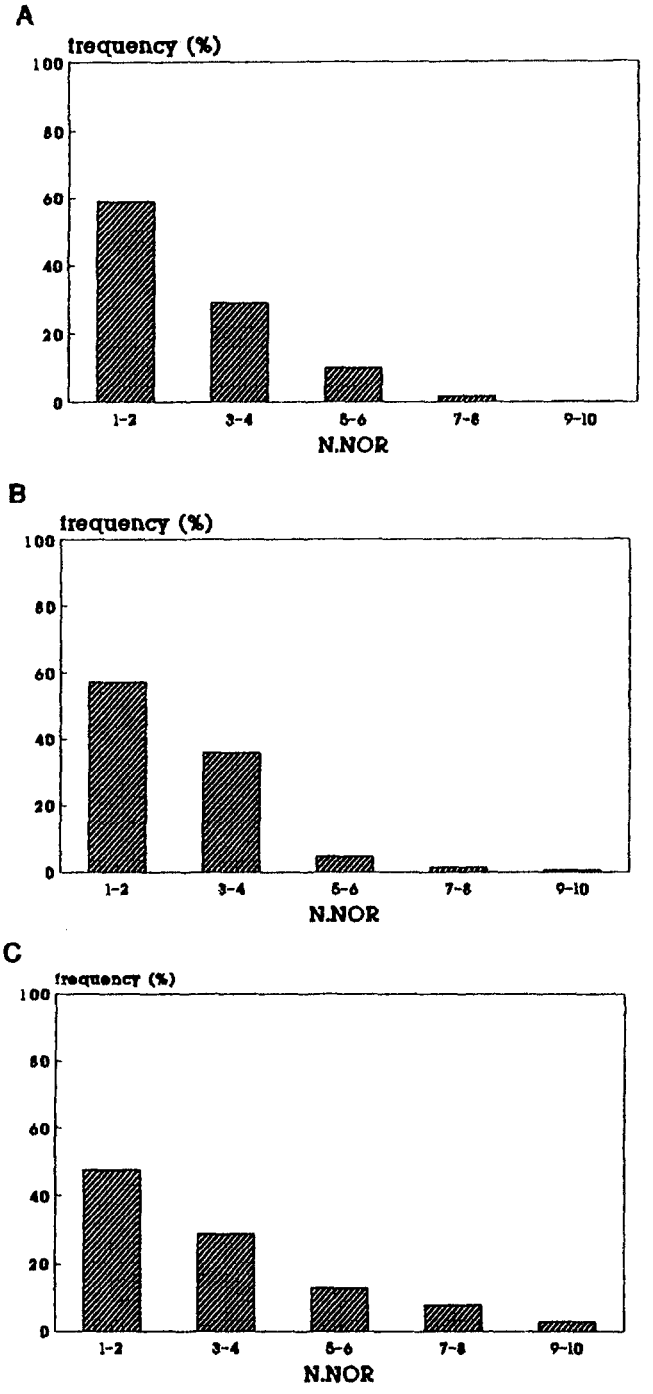

Fig. 3 Frequency distribution of number of AgNOR per nucleus (N NOR) for control epithelia (*A*) and treated epithelia at 17 weeks (*B*) and 24 weeks (*C*) of promotion. Note that a statistically significant shift towards larger values occurs at week 24 ($P<0.01$)

on the dorsal area of initiated animals produced a slight hyperplasia of the epidermis in agreement with Slaga et al. [37] and Klein-Szanto and Slaga [23].

The first tumours appeared on the treated area at 20 weeks. The analysis of this neoplastic tissue revealed the development of papillomas, keratoacanthomas and squamous cell carcinomas.

Morphometric study of AgNOR related variables revealed variations in AgNOR at different time points of chemical carcinogenesis (Fig. 1). The number of AgNOR per nucleus (N.NOR), parameter classically considered in the literature, underwent a significant ($P<0.01$) rise in tumours (in keeping with reports on several human malignant entities) (Fig. 2). No significant differences in N.NOR of skin without tumours were detected during the promotion period until week 24 when tumours have already appeared (Fig. 3).

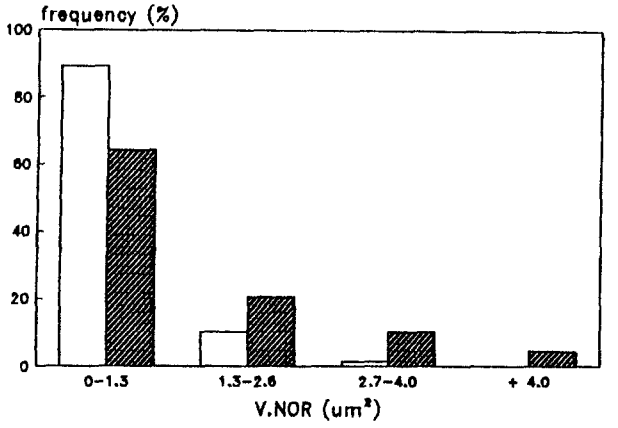

Fig. 4 Frequency distribution of single AgNOR volume (V NOR) for control epithelia (□) and promotion epithelia (▨). Note that a statistically significant shift towards larger values occurs in promoted epithelia ($P<0.01$)

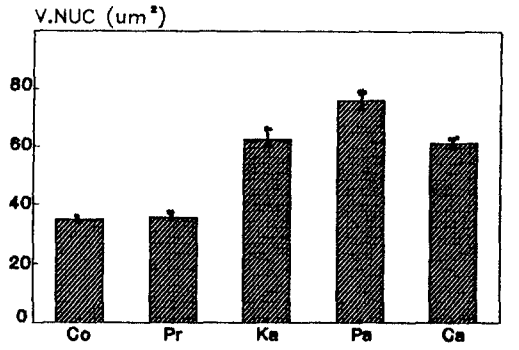

Fig. 6 Average nuclear volume (V NUC) ± standard deviation for different experimental conditions. Note that V NUC failed to vary in promoted epithelia as compared to control epithelia. *Co:* control, *Pr:* promotion, *Ka:* keratoacanthoma, *Pa:* papilloma, *Ca:* carcinoma

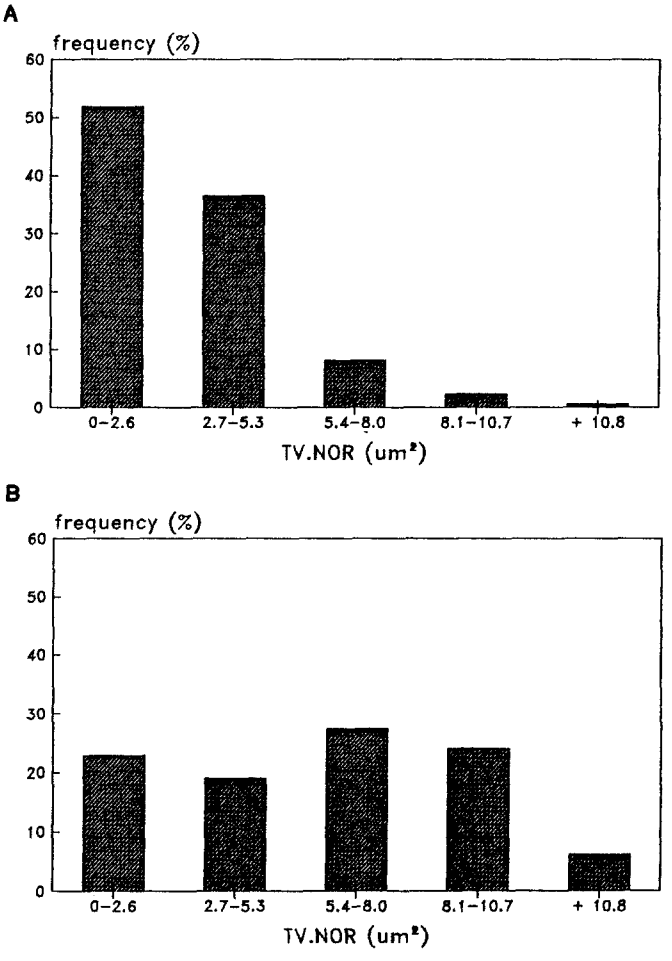

Fig. 5 Frequency distribution of total AgNOR volume per nucleus (TV NOR) for control epithelia (*A*) and epithelia at 2 weeks of promotion (*B*). Note a statistically significant shift towards larger values as early as 2 weeks after the onset of promotion ($P<0.01$)

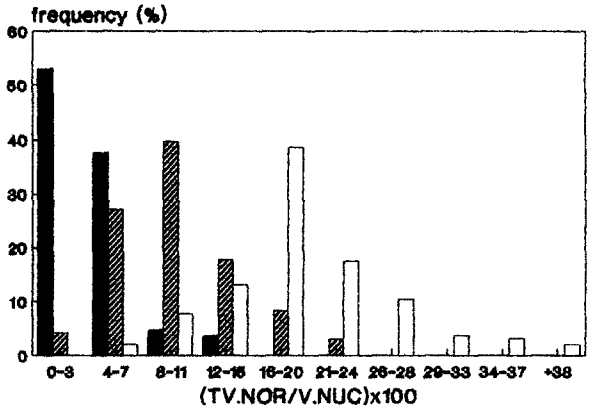

Fig. 7 Frequency distribution of the proportion of nucleus occupied by AgNOR (TVNOR/VNUC) for control epithelia (■), a pool of promoted epithelia (▨) and papilloma (□) as an example of tumour tissue

Average single AgNOR volume (V.NOR) frequency distribution varied significantly exhibiting a shift towards larger values during the promotion period (Fig. 4). Concomitantly, the frequency distribution of total AgNOR volume per nucleus (TV.NOR), revealed a tendency towards greater values during the promotion period, which was statistically significant ($P<0.01$) at week 2 (Fig. 5). Ninety percent of control nuclei featured TV NOR values between 0–5.3 μm^2. During the promotion period a shift towards greater TV.NOR values occurred: only 40–50% of nuclei exhibited values in the 0–5.3 μm^2 range, whereas the rest reached values in excess of 5.4 μm^2 (Fig. 5).

The proportion of nucleus occupied by AgNOR (TV.NOR/V.NUC) increased concomitantly with TV.NOR given that no significant differences in nuclear volume between initiated and promoted skin as compared with all the types of controls were detected (Fig. 6). Nuclear volume values increased significantly ($P<0.01$) in all 3 types of tumours (Fig. 6). VTNOR/VNUC exhibited a shift towards larger values, despite the increase in size of tumour nuclei (Fig. 7).

Single V.NOR and total TV.NOR values in tumours taken together differed significantly ($P<0.01$) from control and promotion values (Fig. 8). No statistically significant differences in V.NOR and TV.NOR were detected among tumour types.

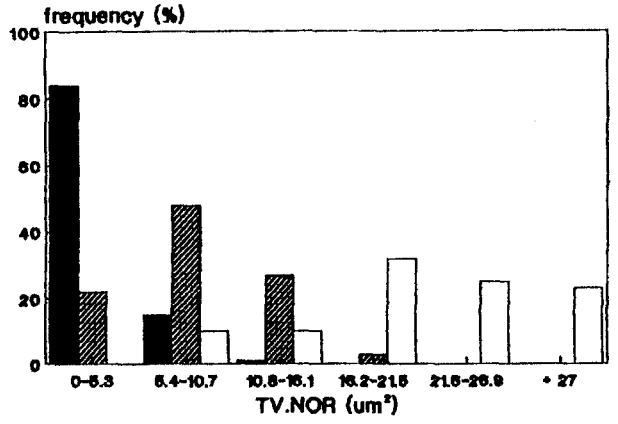

Fig. 8 Frequency distribution of total AgNOR volume per nucleus (TV NOR) for control epithelia (■), a pool of promoted epithelia (▨) and papilloma (□) as an example of tumour tissue

Discussion

The value of AgNOR as a quantitative marker of incipient cellular alterations [35] and malignant transformation [8, 27, 34] suggested its potential use in assessing variations during the period prior to the development of tumours. Thus the promotion period in a model of experimental multistage carcinogenesis was examined as compared to the control and tumour tissue at both extremes of the process.

Evaluation of unconventional AgNOR-related variables has proved of additional or even greater discriminative value than the traditional AgNOR number [1, 5, 11]. Thus, the present study involved a morphometric analysis of AgNORs in addition to AgNOR counting. The analysis of initiated and non-initiated controls revealed no differences in AgNOR variables, showing that the initiation process alone had no effect on AgNOR. Moreover, controls treated with acetone showed no differences from untreated controls. Thus, the alterations in AgNOR are apparently due to the effect of the promotion process on cell activity, in particular, on cellular proliferation and/or transcriptional activity. AgNOR counts have been reported to vary alongside Ki-67 immunoreactivity [17, 29], length of interphase [26] and rRNA transcription [6, 31, 39].

The traditional N.NOR exhibited significant differences ($P<0.01$) between tumours (papillomas, keratoacanthomas and carcinomas) and controls, in agreement with others [5, 13, 27, 30]. However, during the promotion process there was no significant increase in N.NOR up to week 24.

A shift towards larger values of single AgNOR volume in tumour tissue would apparently differ from the tendency to smaller average single AgNOR volume reported for other tumours [1, 5]. The analysis of the present results in terms of frequency histograms reveals the existence of both small and large AgNOR in tumour tissue. Mean values reported in other studies may be masking the existence of two distinct size groups of AgNOR. However, the present results do not rule out the possibility of a different behaviour in terms of AgNOR volume for this model.

Given that the number of AgNOR can increase as a result of formerly inactive NOR or decrease as a result of an association of active NOR [39], AgNOR volume (V.NOR, TV.NOR) would be a more indicative variable.

Significant differences between promoted animals and controls ($P<0.01$) were observed from the second week of promotion when the variables analysed were V.NOR and TV.NOR. These results indicate that volume related measurements are earlier markers of variation in cellular activity than AgNOR counts.

The present results reveal an overall increase in AgNOR material, statistically significant as early as 2 weeks after the onset of promotion, when the tissue still fails to exhibit any unusual microscopic features. The possibility of detecting and evaluating incipient cellular alterations with a simple technique may prove of value.

Interphase NOR can be considered to represent a morphofunctional unit in which all the substances necessary for transcription of ribosomal genes are present [11]. The AgNOR proteins represent a set of acidic proteins that are selectively located in the NOR [28, 31, 34]. At present, their identity is still not established. Several proteins have been proposed as molecules responsible for silver staining, such as the large subunit of RNA polymerase I [41], B23 or numatrin [14, 28] and C23 or nucleolin [28, 31, 38]. The accumulation of B23 is closely correlated with entry and progression through S phase, thus, the increase in B23 would be associated with induction of mitogenesis [14, 15, 16]. C23 would control the rate of ribosomal gene transcription [25]. Thus, the increase in C23 and RNA polymerase I would be correlated with transcriptional activation of rRNA genes. Given that C23 protein may be the main silver-staining protein [31], the increase in VNOR and TVNOR would indicate transcriptional activation of previously inactive NOR, leading to a rise in rRNA synthesis and, ultimately, in protein synthesis.

These events would correlate with the gene activation phenomena which occur during promotion and which in turn lead to expression of the altered phenotype.

The present evidence would suggest the potential value of AgNOR-related variables to detect and evaluate cellular alterations at a very early stage.

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