# The deazaguanine-derivative, queuine, affects cell proliferation, protein phosphorylation and the expression of the proto oncogenes c-*fos* and c-*myc* in HeLa cells

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In higher eukaryotes the hypermodified guanine analogue, queuine (7-(5-(((1S,4S,5R)-4,5-dihydroxy-2-cyclopentene-1-yl)amino)-methyl)-7-deazaguanine), occurs free or as modified nucleoside (Q) in the anticodon of specific tRNAs. Fast proliferating tissues and tumors contain considerable amounts of free queuine and Q-deficient tRNAs. Here we show that HeLa cells can be grown in the absence or presence of queuine. In response to queuine, and under appropriate conditions, (i) the proliferation of HeLa cells was stimulated, (ii) the steady-state level of c-*fos* mRNA was reduced, contrary that of c-*myc* mRNA was elevated, and (iii) in cytosolic extracts protein phosphorylation especially for a 42 kDa protein was significantly increased. The results suggest that queuine substitutes for growth factors in a signal transduction pathway.

Queuine; Growth control; Signal transduction; Oncogene; Protein phosphorylation

## 1. INTRODUCTION

The modified base, queuine (7-(5-(((1S,4S,5R)-4,5-dihydroxy-2-cyclopentene-1-yl)amino)-methyl)-7-deazaguanine), cannot be synthesized by mammals, but is obtained from their diet or gut flora (for summarizing references see [1,2]). Mammalian cells are supplied with queuine by fetal calf serum when grown in culture [3]. Queuine is inserted into position 34 (wobble base) of the anticodon of tRNAs accommodating for the amino acids asparagine, aspartic acid, histidine and tyrosine [4]. This insertion is mediated by the eukaryotic tRNA-guanine transglycosylase (EC 2.4.2.29) in a base for base exchange reaction with guanine [5]. The occurrence of queuine and of queuosine tRNAs is highly conserved from eubacteria to eukaryotes, yeast being the only known exception. Alterations of the metabolism of queuine are tissue specific and are dependent on the metabolic state of cells and tissues. Alterations in the queuine-modification of tRNAs and fluctuations in the amount of free queuine in eukaryotic cells occur especially during development and differentiation [6]. In neoplastically transformed, fast dividing cells free queuine and Q-deficient tRNAs accumulate. Nishimura was the first who surmised that the presence of Q-deficient tRNAs in tumor cells is not the result of their proliferative state, but rather a specific property of tumors and undifferentiated cells [1]. Inhibition of queuine modification of tRNAs has been implicated in the promotion of carcinogenesis in vitro [7]. For human lymphomas we have found that the relative amount of Q-deficient tRNAs is generally greater in high grade than in low grade lymphomas; these lymphomas contain relatively high amounts of free queuine and Q-deficient tRNAs. Both the accumulation of Q-deficient tRNAs and of queuine are correlated with the proliferative activity of the lymphoma tissue [8]. Deranged proliferation during carcinogenesis appears to involve abnormal signal transduction pathways [9,10]. We have therefore established HeLa cells as a model system to investigate whether queuine affects cell proliferation and related processes such as the expression of oncogenes and specific protein phosphorylations. The present data support this view.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Eagle's minimum essential medium, modified for autoclaving (MEM) was from Gibco/BRL Laboratories (Eggenheim, FRG). Horse serum was from Boehringer Mannheim (FRG) and was analyzed for queuine-deficiency [6]. It was essentially queuine-free. HeLa-S3 cells were kindly supplied by Dr Ogilvie (author's address). Plasmids, carrying the c-*fos* or c-*myc* oncogenes (Amprobe) were from Amersham Corp. (UK). Protease inhibitors, leupeptin, pepstatin, aprotinin and $\alpha_2$-macroglobulin were from Boehringer Mannheim (FRG).

### 2.2. *Cultivation of cells*

HeLa-S3 cells were routinely grown in 10 ml MEM medium, supplemented with 10% horse serum, in culture dishes of 8.5 cm in diameter. Incubation was in an atmosphere of 5% $CO_2$ and 95% air. Queuine was added to a final concentration of $3.6 \times 10^{-7}$ M. For

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determination of the cell number, cells were collected by trypsinisation and counted in a Fuchs/Rosenthal chamber.

2.3. *Preparation of RNA and Northern blot analysis*

Total RNA was extracted from $1-2 \times 10^7$ cells by the guanidinium thiocyanate procedure, modified as follows: cells were first suspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.65% NP-40) [11] and kept on ice for 15 min. Cells were then lysed on a vortex mixer for 1 min or by pipetting them up and down several times. Cell debris and nuclei were removed by centrifugation at $3000 \times g$ for 10 min. The supernatant was subjected to the guanidinium thiocyanate procedure, omitting the protease treatment. RNA was separated in 1% agarose/formaldehyde gels and blotted onto GeneScreen plus (New England Nuclear) according to standard procedures. Filter-bound RNA was hybridized to a nick-translated plasmid carrying part of either the c-*fos* or the c-*myc* gene.

2.4. *Phosphorylation assay*

$1-1.5 \times 10^7$ cells were collected from monolayer cultures and lysed in hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 7 mM $\beta$-mercaptoethanol) in the presence of protease inhibitors (1 $\mu$g/ml each of pepstatin, leupeptin, aprotinin and $\alpha_2$-macroglobulin). Cytosolic proteins were obtained by graduate centrifugation as described in [12]. For in vitro phosphorylation, 200 $\mu$g of cytosolic proteins were suspended in a final volume of 40 $\mu$l hypotonic buffer. All subsequent steps were performed on ice. 5 $\mu$l of cycloheximide (final concentration 10 $\mu$g/ml) and 5 $\mu$l of 0.1 M $MgCl_2$ was added. Labeling was started by the addition of 10 $\mu$Ci [$\gamma$-$^{32}$P]ATP (3000 mCi/mmol) and stopped after 10 min by adding 6 $\mu$l 0.2 M EDTA. One-half of the sample was subjected to 12.5% SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie blue, destaining, drying and autoradiography.

## 3. RESULTS

### 3.1. *Stimulation of cell proliferation*

HeLa cells were grown in media supplemented with queuine-free horse serum in monolayer cultures without (−Q) or with added queuine (+Q) at a concentration of $3 \times 10^{-7}$ M. Obviously the total amount of cells per culture dish was about 2-fold higher in +Q-cultures than in −Q-cultures. When queuine was added to the −Q-culture after cell division had ceased, the cells started to divide again and also reached the final cell density per culture dish that corresponded to the +Q-culture (Fig. 1). Under the conditions described, queuine acted as a proliferation-stimulating agent on HeLa cells. Similar results can be obtained in the absence of serum factors (not shown).

### 3.2. *Expression of the cellular proto-oncogenes* c-fos *and* c-myc

The cellular proto-oncogenes, c-*fos* and c-*myc* are believed to be important in cell cycle control mechanisms and in the mitogenic response [10,13]. We reasoned that the stimulating activity of queuine on the proliferation of HeLa cells might be accompanied by changes in the expression of these two genes. Therefore total RNA was extracted from HeLa cells, which were grown in the presence or absence of queuine. After electrophoresis the c-*fos* and c-*myc* mRNA levels were investigated by Northern-blot analysis. The results show that +Q-cells contained a significantly lower level

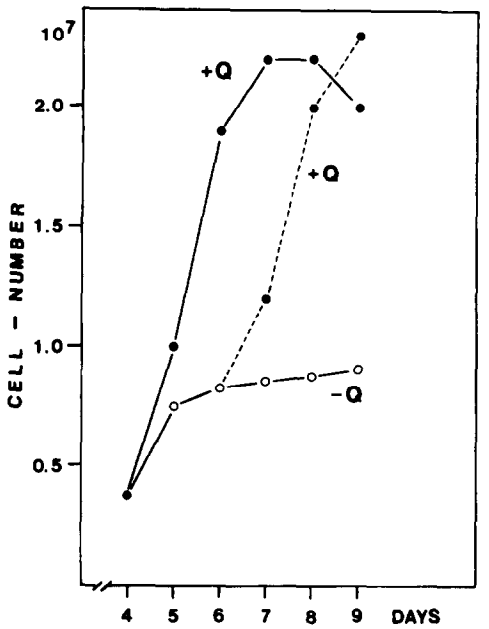


Fig. 1. Growth of HeLa cells in the presence (+Q) or absence of queuine (−Q). $5 \times 10^5$ cells were seeded to each culture dish and incubated for 3 days without queuine. On the fourth day the medium was changed and one portion of the cultures received queuine (•), while the other portion did not (○). On the sixth day an additional portion of previously queuine free cultures was supplied with queuine (- - -). The medium was changed on the seventh day.

of c-*fos* however, a significantly higher level of c-*myc* mRNA than −Q-cells (Fig. 2). Since +Q- and −Q-cells were grown under identical conditions, the altered levels of c-*fos* and c-*myc* mRNA can be traced back exclusively to the presence or absence of queuine in the growth medium.

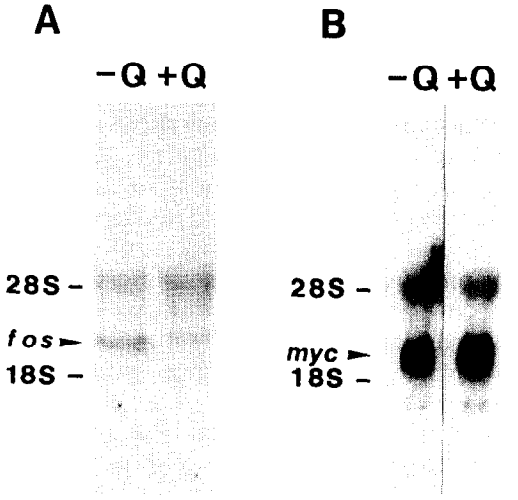


Fig. 2. Queuine-dependent expression of the c-*fos* and c-*myc* genes in HeLa cells. $1 \times 10^6$ cells were seeded to each culture dish and incubated either with (+Q) or without queuine (−Q) for 4 days with exchange of the medium on the second day. RNA was extracted and 40 $\mu$g each were separated by electrophoresis followed by Northern-blot analysis (see section 2). Filter-bound RNA was hybridized to labeled plasmids carrying either the c-*fos* (A) or the c-*myc* gene (B).

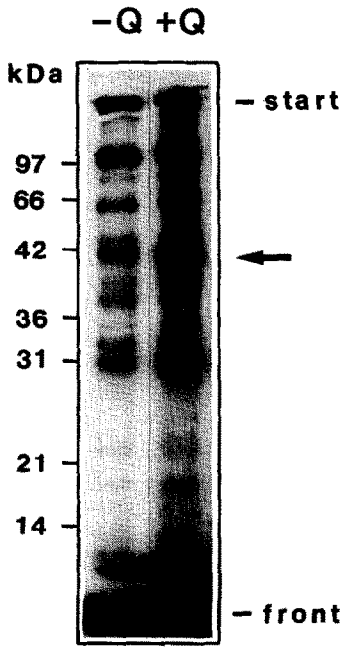


Fig. 3. In vitro phosphorylation of cytosolic proteins from HeLa cells grown with (+Q) or without queuine (−Q). HeLa cells were grown in this experiment as described in the legend to Fig. 2. Cell-fractioning, $^{32}$P-labeling and electrophoresis was done as described in section 2. The autoradiography of the dried gel is shown. The arrow (←) indicates a protein specifically labeled in (+Q) cells.

### 3.3. *Protein phosphorylation*

The signaling pathway that leads from the binding of growth factors to the c-*fos* and c-*myc* gene activation and subsequent cellular responses involves a complex network of protein phosphorylations [9,10]. Therefore we have studied protein phosphorylation by using cytosolic extracts from +Q- and −Q-cells. The extracts were incubated with [$\gamma$-$^{32}$P]ATP at 0°C for 10 min and the labeled proteins were analyzed by one-dimensional electrophoresis. Indeed specific, queuine-dependent alterations in protein phosphorylation were observed (Fig. 3). Especially one protein with a molecular mass of about 42 kDa became heavily phosphorylated in extracts from +Q-cells, but much less in extracts of −Q-cells.

## 4. DISCUSSION

The growth of HeLa cells in culture depends on the availability of growth factors in the medium. These growth factors bind to specific receptors on the cell surface which in turn leads to a signal that is transduced into the cell [10]. The earliest cellular responses that can be observed are: (i) the activation of protein kinases, especially protein kinase C; and (ii) the transient and rapid increased expression of the primary response genes c-*fos* and c-*myc* [10,13]. This is followed by the cellular response, i.e. initiation of DNA synthesis and submission to the cell cycle. Any alterations in this signal transduction pathway have been linked with cancer. A more relaxed control of cell proliferation and a reduced dependency on growth factors is typically observed with tumor cells. The finding that queuine can stimulate proliferation suggests that it may interfere with or participate in this signaling pathway that activates the mitogenic potential of the cell. It appears as if queuine stimulates or even can mimic the effect of growth factors on cell proliferation. This stimulatory effect coincides with a queuine-dependent modulation in the expression of the proto oncogenes c-*fos* and c-*myc* in the growing cells. These two genes are assumed to be primary targets of the signaling pathway initiated by growth factors and other mitogens such as phorbol esters and are important in the cell cycle control [9,10,13]. It seems likely that the proliferation-stimulating activity of queuine is mediated primarily by the differential expression of these two genes. The reduced level of the c-*fos* mRNA in +Q-cells might be caused by an increased translation resulting in a destabilization of this RNA [15]. The specific effect of queuine on protein phosphorylation can be taken as further evidence for the involvement of queuine in cellular signaling. The phosphorylation of a 42 kDa protein in response to mitogenic stimuli has indeed been reported [14].

As a consequence of our findings we propose that the eukaryotic nutrient factor queuine is involved in intracellular signaling and may indeed play a role in the generation or maintenance of malignant transformation.

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