Transient transcriptional inhibition of the transferrin gene by cyclic AMP

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Transferrin mRNA content and gene transcription rate were measured in the liver of rats submitted to iron overload or depletion, castration, treatment with sexual steroid hormones, glucagon and cyclic AMP. The influence of puberty in males and females and of pregnancy was also analysed. Glucagon and cyclic AMP reduced mRNA level by about 50% at the 12th hour of treatment and transferrin gene transcription by as much as 95% at the 30th minute of drug infusion, with a secondary increase of the transcription rate for a protracted treatment. None of the other hormones tested had any detectable effect on transferrin gene expression, the same being true for iron overload or depletion.

Transferrin gene *Transcription* *Cyclic AMP* *Iron overload* *Iron depletion* *Steroid hormone*

1. INTRODUCTION

Cyclic AMP (cAMP) has recently been proved to be able to transcriptionally inhibit the expression of several genes encoding carbohydrate-stimulated liver enzymes [1,2] and is known since 1982 to be a transcriptional activator of other genes, especially liver genes whose expression is repressed by carbohydrates and insulin [3,4].

We show in this paper that the transferrin gene, encoding serotransferrin, the main iron-binding protein in serum, is itself modulated by cAMP: transcription of the gene is profoundly but transiently inhibited by both cAMP and glucagon, while other agents which were expected to regulate transferrin gene expression (iron and sexual steroid hormones) proved to be without any detectable influence on the gene transcription.

2. MATERIALS AND METHODS

2.1. *Animals*

The following adult animals weighing 200–250 g were used: (i) 6 male Wistar rats were made anaemic by cardiac puncture, 1 ml twice a week, and by feeding an iron-deficient diet (gruyere cheese and egg white). Rats were killed after 5, 7, 8, 9 and 10 cardiac punctures. Hyposideraemia was profound from the 5th puncture (serum iron concentration $< 5\ \mu M$, normal range $40 \pm 5\ \mu M$). Serum transferrin level was measured by the iron capacity saturation which was $> 160\ \mu M$ (normal range $106 \pm 20\ \mu M$). (ii) Iron overload was produced in 18 Sprague Dawley male rats over a 3-month period by using the method of Bacon et al. [5] slightly modified as follows: in our protocol, rats weighing initially 200 g were fed a diet supplemented with 5% carbonyl iron. This model produces a pathological pattern of iron overload similar to human haemochromatosis (marked hepatocellular iron overload with a periportal distribution). (iii) 24 Sprague Dawley rats (12 males, 12 females) were castrated. 9 females were given 20 μg oestradiol in a single intradermal injection [6,7]; 3 of them were killed 2, 5 and 12 h after hormone injection. Oestrogen effect on ovariec-

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tomized rats was assessed by weighing the uterus and measurement of uterine progesterone receptors [8]. In all cases oestrogen treatment caused an increase in uterine weight and in the number of uterine receptors showing that the treatment was effective [8]. The same procedure was repeated with 9 castrated male rats injected with 1 mg testosterone. 3 castrated rats of each sex were killed before any hormonal treatment.

The influence of glucagon and cAMP was studied in Wistar rats exactly as published [1].

A rat transferrin cDNA probe about 1400 bp long was used in this study [9,10]. The methods of RNA purification, Northern and dot blot analysis have been described [1,10,11].

2.2. *Run-on transcription on isolated nuclei*

Transcription by isolated nuclei and subsequent isolation of ^{32}P-labeled RNA were performed as described [2]. Briefly, nuclei were isolated from rats submitted to the studied metabolic conditions and the RNA polymerase bound on the gene at the moment of killing allowed to continue RNA synthesis in the presence of radioactive precursors.

The reaction was terminated by the addition of DNase I made RNase-free by a 2-h treatment at 37°C with proteinase K in the presence of $CaCl_2$ [12]. The radioactively labeled nuclear RNAs were purified by ethanol precipitation in guanidine-HCl in the presence of carrier poly(A^-)-RNA from rabbit muscle, then hybridized to transferrin cDNA sequences immobilized on 'gene screen plus' nylon filters: this type of nylon membrane allows multiple use of the specific filters without decrease of the specific signal (unpublished).

After extensive washing and digestion with pancreatic RNase, the fraction of the total radioactive transcripts derived from the exonic transferrin gene sequence which hybridizes with cDNA probe was measured by liquid scintillation counting.

Results are expressed in parts per million of radiolabeled RNA (ppm).

Rate of transferrin mRNA synthesis (in ppm) = specific cpm eluted from the filters/total cpm of radiolabeled RNA × (100/efficiency) × the length of transferrin gene/the length of the transferrin cDNA insert in base pairs. The length of the transferrin gene has been recently estimated to be about 30 kb [13]. To estimate the hybridization efficiency, [^{3}H]cRNA was prepared according to Lis et al. [14] using the purified 1400 bp cDNA insert: hybridization efficiency was on average 40%.

Typically, 25–40 × 10^6 cpm of labeled RNA were obtained from each reaction. Background hybridizations to a pBR322 filter were less than 20 ppm.

The DNA of a recombinant plasmid containing sequences complementary to mRNA for the albumin protein was included as an internal standard in the hybridization experiment. The length of the albumin gene is 15 kb and the cDNA insert is 1000 bp [15].

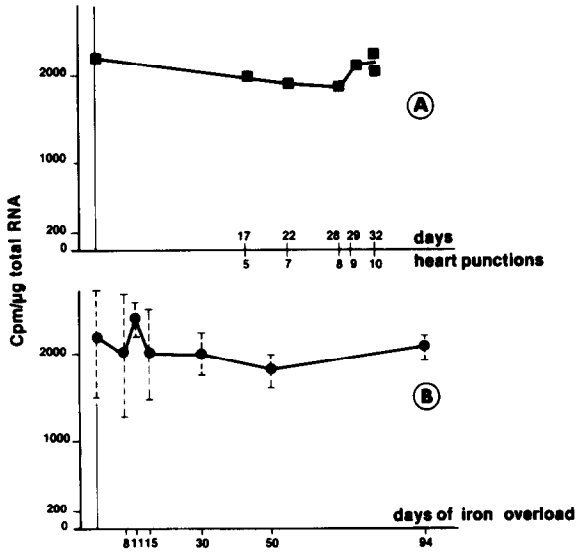

Fig.1. Influence of iron on liver transferrin mRNA content. For measuring specific mRNA concentration by dot blot analysis, 10 µg and 5 subsequent 1:2 dilutions of total RNA (and poly A^+ mRNA for control) were dotted onto nitrocellulose filters, the hybridization being performed in the presence of 2 × 10^6 cpm/ml of labeled purified inserts (0.5 ml of hybridization mixture per 10 cm^2 of filter surface). The radioactivity was a linear function of the RNA amounts deposited on the filters. The results are expressed in cpm of radiolabeled probe per µg of RNA. All the individual experiments included a standard range consisting of the aliquoted dilutions of the same normal liver RNA preparation. In preliminary hybridization experiments transferrin mRNA of this standard preparation was measured to 2200 cpm/µg RNA. All the results were normalized with respect to this value. (A) Iron deficiency induced by heart punctures. Each point represents the result of one individual measurement. (B) Iron overload. Each point represents the mean ± 1 SD of 6 determinations in each of 3 rats.

Table 1

Effects of castration, oestrogen administration and iron status on transferrin gene transcription

Hormonal status	Transferrin gene transcription	Albumin gene transcription
None	811 ± 49	1208 ± 233
Castration (male)	771	1155
Castration (female)	772	1192
Oestradiol administration	1170	1035
Iron overload [1]	1086 ± 53	1404 ± 240
Iron deficiency [2]	943 ± 80	1380 ± 150

Relative rate of transferrin and albumin gene transcription was measured in isolated liver nuclei from animals under various hormonal conditions. For iron deficiency and iron overload, results are means ± 1 SD of 6 different determinations performed in 3 animals (2 per animal). For castration and influence of oestradiol, results are for single animals. The results are expressed in part per million (ppm) of specific transferrin mRNA

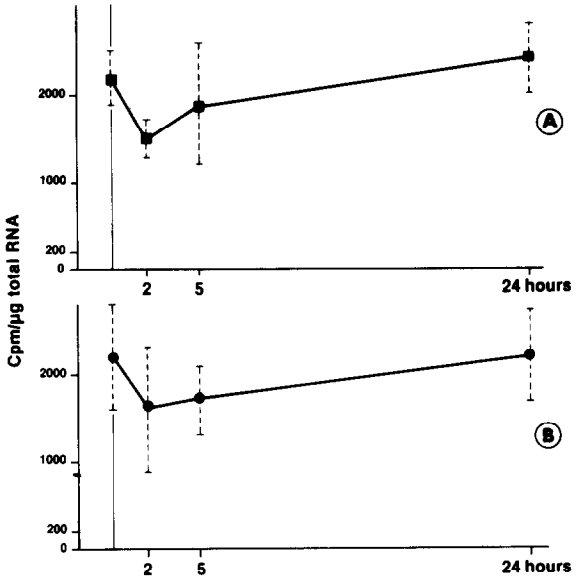

Fig.2. Influence of oestradiol and testosterone on liver transferrin mRNA content. (A) 1 mg testosterone was injected into castrated male rats at time zero. (B) 20 µg oestradiol were injected into castrated female rats at time zero. Legend and symbols similar to fig.1.

3. RESULTS

3.1. *Iron deficiency and overload*

Fig.1 and table 1 show that no significant reproducible modification of transferrin mRNA content and gene transcription could be observed either in rats overloaded with iron or in anaemic, iron-deficient animals.

3.2. *Influence of sexual steroid hormones*

In the same way, fig.2 and table 1 indicate that transferrin gene expression was not affected by either oestrogens or testosterone. These results are in agreement with those obtained in two pregnant female rats (transferrin mRNA = 2180 and 2300 cpm/µg RNA) and with those we have recently published using pre- and post-pubescent males and females: indeed, transferrin mRNA concentration in the rat liver is practically stable from birth to the middle of life [9].

3.3. *Influence of glucagon and cyclic AMP*

In fig.3, it can be observed that, in contrast, treatment for 12 or 24 h with dibutyryl cAMP or glucagon resulted in a decrease of about 50% in

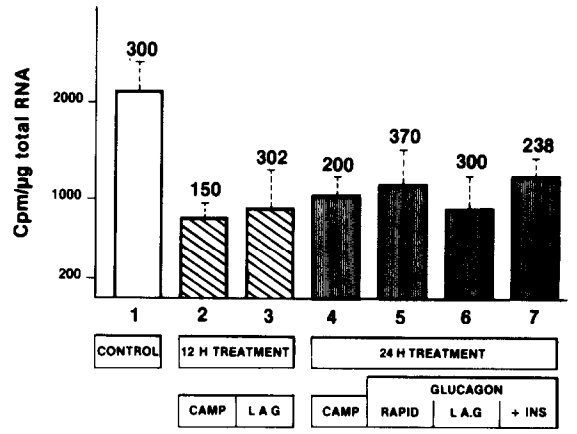

Fig.3. Influence of dibutyryl cyclic AMP and glucagon on liver transferrin mRNA content. (1) Control. (2,3) 12 h treatment. (2) cAMP, 0.2 mg/kg per h; (3) long-acting glucagon, 100 µg/12 h. (4–7) 24 h treatment. (4) 0.2 mg/kg per h cAMP; (5) 0.5 mg/kg per h rapid glucagon; (6) long-acting glucagon, 200 µg/24 h; (7) glucagon + insulin, 200 µg/24 h and 5 units regular and long-acting insulin, respectively. Each value represents the mean ± SD of 6 determinations in each of 3 rats.

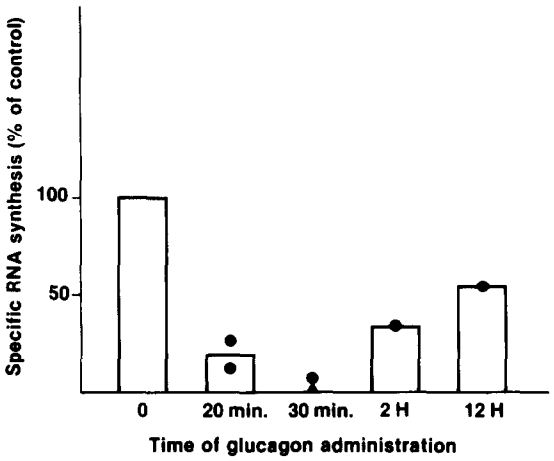

Fig.4. Influence of glucagon and dibutyryl-cyclic AMP on the transcription rate of the transferrin gene. Glucagon (12.5 μg/kg of body wt) was injected intravenously (animals) during 20 min, 30 min and 2 h. Long-acting glucagon was injected at the 12 h point [1]. Dibutyryl cyclic AMP (25 mg/kg of body wt) was injected intraperitoneally. (●) Glucagon, (▲) dibutyryl cyclic AMP. Transferrin gene transcription was measured with the nuclear run-on transcription assay as indicated in section 2. Each individual value is expressed as a percentage of the mean transferrin gene transcription in untreated rats.

transferrin mRNA; the effect of glucagon could not be reversed by insulin administered together with glucagon.

The influence of glucagon and cAMP on the transcription rate of the transferrin gene is shown in fig.4. Glucagon infusion induced a rapid and specific inhibition of the transferrin gene transcription to less than 5% of the initial rate at the 30th min of treatment. Exactly the same result was obtained with dibutyryl cAMP. When glucagon infusion was prolonged for 2 h, a reinitiation of transferrin gene transcription could be observed; this result was confirmed in the animals treated with long-acting glucagon for 12 h. Such reinitiation of the transcription of genes negatively regulated by cAMP upon protracted treatment with glucagon cannot be explained by a desensitization of the hormone receptors because other genes, such as the pyruvate kinase gene, remain transcriptionally blocked under the same conditions [2].

Inhibition of gene transcription by cAMP is obviously neither a general phenomenon [4] nor an experimental artifact since the albumin gene transcription rate studied as a control under the same conditions is not affected [2].

4. DISCUSSION

Our results that sexual steroid hormones and iron status do not modulate transcription of the transferrin gene and specific RNA content are slightly at variance with the data of McKnight et al. [16,17] concerning regulation of the ovotransferrin gene in the chick liver: these authors reported transcription of the gene to be stimulated 1.5–2-fold by both iron deficiency and oestrogens. Chambon et al. [18], in contrast, were unable to confirm the regulation of this gene by oestrogens in the chick liver. In any case it appears that regulation of the transferrin concentration in the serum is a function of the iron status, which is a phenomenon known for a long time [19], and is not primarily transcriptional.

In fact, the main finding concerning transferrin gene regulation in the rat is that cAMP induces an almost complete, but transient blockade of gene transcription. From a physiological point of view, it is doubtful whether this phenomenon plays any significant role because transcriptional inhibition, although profound (>95%), is brief compared with the half-life of mRNA and, above all, proteins [mRNA level is not significantly decreased 3 h after cordycepin injection in the rat (S. Vaulont et al., unpublished)]; transferrin half-life is 7–12 days [20,21].

From a theoretical point of view, however, this observation could be important. It is more and more evident that cAMP is frequently involved as a regulator of gene transcription in eucaryotes, sometimes as a transcripotional activator (for instance, in the liver, for the phosphoenolpyruvate carboxykinase [4] and tyrosine aminotransferase [3] genes), and sometimes as a transcriptional inhibitor, as we have recently demonstrated for 3 carbohydrate-induced hepatic genes encoding L-type pyruvate kinase, aldolase B and an unidentified 5.4 kb mRNA species [2]. In these 3 genes transcriptionally inhibited by cAMP, the inhibition persists as long as glucagon or cAMP are administered (Vaulont et al., in preparation). Moreover, endogenous secretion of glucagon induced, for instance, by a protein-rich diet, results

in a blockade of the above gene transcription which can persists several days or weeks and is reversible only when protein administration is discontinued and glucagon secretion decreases (Vaulont et al., unpublished). In contrast, a prolonged protein diet does not decrease transferrin gene transcription at all (Vaulont et al., in preparation).

The mechanism of the transcriptional effects of cAMP remains absolutely unknown, the two generally proposed hypotheses being that of the binding of cAMP to an eucaryotic equivalent of the *E. coli* cap protein [22] and of the phosphorylation of a transcriptionally active factor [23,24].

With regard to this latter hypothesis, it could be suggested that phosphorylation of transcription factors leading to this inactivation involves several classes of genes, some of them being able to specifically escape this inhibition. The molecular basis of this specific escape remains as obscure as that of the primitive action of cAMP; a differential rate of dephosphorylation of those factors active on reversibly or irreversibly inhibited genes could be speculated.

In any case, these results emphasize the complex role of cAMP on eucaryotic gene expression.

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