IN VITRO MOUSE MAMMARY TUMOR VIRUS TRANSCRIPTION FROM CHROMATIN

A system to study the mechanism of action of glucocorticoid hormones

Michel CREPIN

Gordon M. Tomkins Laboratory, Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143, USA

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1. Introduction

The analysis of transcription of a specific mRNA at a nuclear level remains technically difficult because in spite of recent progress in the characterization of messenger RNAs from various animal sources the remainder of eukaryotic genes whose products have been accurately identified is still relatively small. Cellular system infected or transformed with RNA tumor viruses constitute interesting tools to study the control of genes coding for a specific mRNA. RNA tumor viruses are characterized by a large single stranded RNA genome [1] and they contain an RNA-directed DNA polymerase which transcribes the viral genome into single stranded DNA [2,3]. This complementary DNA can be used as a probe to detect specific viral mRNA copies. Moreover non-infected cells of mice might contain the genetic capacity to synthesize C-type [4] or B-type viruses [5]. The presence of virus-specific DNA stably integrated in the genome of these cells, was shown by nucleic acid hybridization [6,7]. Chromatin from these virus transformed systems has been shown to retain its cell-specific template activity [8–10]. Cultured cells derived from mammary carcinomas of mice spontaneously release mouse mammary tumor virus (MMTV), an RNA tumor virus which replicates via a DNA intermediate integrated into the DNA of the host (reviews [11,12]). A cell line isolated from primary explants of mammary tumor in GR mouse contains 70 viral genome equivalents integrated into the cellular genome (Morris, V. personal communication).

Steroid hormones play an important role in regulating viral gene expression. It is generally accepted that the hormone binds to a specific cytoplasmic receptor protein, and the steroid–receptor complex thus formed is transferred to the nucleus where it becomes associated with a nuclear component (review [13]). Yet, the precise nature of the 'target' of the steroid–receptor complex, which appears to be in the chromatin fraction, remains unknown. In order to show the presence of specific binding loci in chromatin it would be necessary to measure a specific messenger synthesized in the initial response to the hormone. Treatment of GR cells with dexamethasone, a synthetic glucocorticoid, increases 10–20-fold the intracellular steady state concentration of MMTV RNA and the production of mature virus particles [14]. Because glucocorticoids have an early effect on MMTV RNA synthesis, this cell line provides a potentially useful system to study the primary effect of hormones on specific gene expression. In the present report we have developed a cell-free transcription system constituted with chromatin from GR cells and purified eukaryotic RNA polymerase B. Using this system we have demonstrated an in vitro MMTV RNA synthesis. Chromatin-directed MMTV RNA synthesis was stimulated by prior treatment of GR cells with dexamethasone.

Present address: Institut Pasteur, Département de Biologie Moléculaire, 28, Rue du Dr Roux, 75724 Paris Cédex 15, France

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2. Materials and methods

2.1. *Preparation of MMTV specific DNA and determination of viral MMTV RNA by hybridization with complementary DNA*

DNA complementary (cDNA) to the MMTV RNA genome was synthesized using the endogenous DNA polymerase activity of the virus purified from a GR cell culture supernatant as described [15]. The cDNA finally prepared had spec. act. 10^8 cpm/μg. The viral RNA concentration within RNA extracted from the chromatin incubation mixture, was determined by annealing increasing amounts of RNA with: 1000 cpm cDNA – at 68°C, in 0.6 M NaCl; 0.05 M Tris HCl, pH 7.4; 0.003 M EDTA. The extent of hybridization was assayed by resistance of labeled cDNA to the single-stranded specific nuclease S_1 [16].

2.2. *Transcription of chromatin and preparation of RNA*

Chromatin was prepared from confluent cultures of GR cells using the technique developed by Biessman et al. [17]. This mostly-soluble chromatin contains endogenous RNA polymerase activity representing about 15% activity measured in nuclei. The standard assay mixture (2 ml) contained: 80 mM Tris–HCl, pH 7.9; 0.1 M NH_4Cl 3 mM $MnCl_2$; 0.4 mM CTP–ATP–GTP–UTP, or 0.1 mM [^{3}H]UTP (spec. radioact. 21 Ci/mM), plus chromatin corresponding to 2 $A_{260\,nm}$ units. The reaction was started by adding 5 units calf thymus RNA polymerase B. Incubations carried out at 30°C for 1 h were terminated by dilution to 10 ml with Tris–HCl, 50 mM, pH 7.6; $MgCl_2$, 10 mM and cooling in ice. Worthington RNAase-free DNAase, (20 μg/ml), was rapidly added and incubation was continued for 1 h at room temperature. The incubation mixture was treated with 500 μg/ml pronase and 0.5% (w/v) SDS for 1 h at 37°C. Nucleic acids were extracted twice with phenol and precipitated with ethanol. Pellets of nucleic acids resuspended in 10 mM Tris, pH 7.6, plus 10 mM $MgCl_2$ were treated with DNAase for 2 h at room temperature. The RNA was re-extracted with phenol, precipitated with ethanol and resuspended in small vol. 10 mM Tris, pH 7.6, 3 mM EDTA to a concentration of about 3 mg/ml.

3. Results and discussion

3.1. *Detection of MMTV RNA sequences transcribed from the chromatin of GR cells*

Chromatin isolated from GR cells was transcribed with either endogenous RNA polymerase or calf thymus eukaryotic RNA polymerase B. The concentration of MMTV RNA present in the RNA extracted from the transcription incubation mixture can be determined by measuring the extent of hybridization between an excess of RNA and the labeled complementary viral DNA (cDNA). The percent cDNA hybridization to pure viral RNA can be calculated from the curve 'e' in fig.1 ($crt_{1/2} = 2 \times 10^{-2}$ mol–s/liter). Thus the concentration of viral RNA in the samples can be estimated from the RNA concentra-

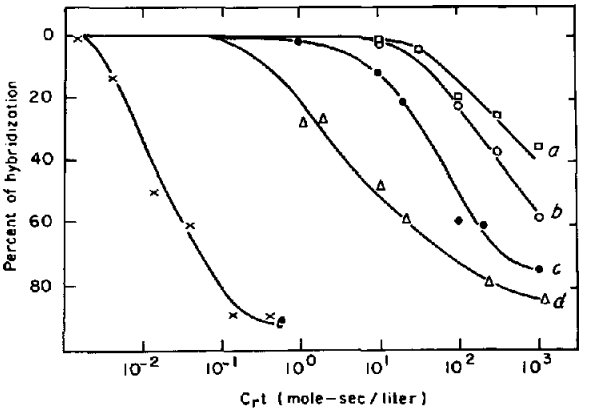

Fig.1. Transcription of MMTV RNA with calf thymus RNA polymerase B. As described in Materials and methods 80 μg chromatin from GR cells treated with dexamethasone (10^{-6} M) was prepared and transcribed with RNA polymerase B (△), or without enzyme (○). Approx. 1000 cpm of MMTV cDNA were annealed with increasing amounts of total RNA or MMTV RNA for 28 h at 69°C in 0.6 M NaCl. Viral particles were purified and MMTV RNA extracted under conditions described in Materials and methods.

(a) RNA from chromatin incubated without XTP
(b) RNA from unincubated chromatin
(c) RNA from incubated chromatin
(d) RNA transcribed with RNA polymerase B
(e) MMTV RNA purified from viral particles

Percent of hybridization was corrected for non-specific annealing and represents the ratio (percent measured – percent annealing with sperm DNA/ (100% annealing with sperm DNA).

tion and the time necessary for annealing of cDNA [18]. As a function of *crt* (concentration of RNA × time of hybridization) the percentage of hybridization for RNA extracted from chromatin unincubated (b) or incubated in the presence (c) and in the absence (a) of nucleoside triphosphates is shown in fig.1. The shift between the *crt* curves 'd' and 'a' or 'd' and 'b' measures the MMTV RNA synthesized in vitro by endogenous RNA polymerase associated with chromatin. The relatively high amount of endogenous MMTV RNA in the control samples (a, b) can be explained in two different ways:

(i) This RNA represents endogenous RNA associated with chromatin.

(ii) Contamination by cytoplasmic MMTV RNA could occur during preparation of chromatin.

Under steady state conditions of induction by dexamethasone, 0.008% and 0.4% MMTV RNA can be detected, respectively, in nuclei and cytoplasm (unpublished results). The relatively high amount of MMTV RNA in nuclei (0.008%) compared to that of the chromatin (0.002%) (table 1) strongly suggests that this RNA represents endogenous nuclear MMTV RNA. For the sake of the results presented here, the important point in any case is that this MMTV RNA concentration is much less than the concentration found after transcription by endogenous or exogenous RNA polymerase.

Ringold et al. [14] have shown that the kinetics of hybridization of MMTV cDNA to MMTV RNA are slightly modified by large variations of the RNA/DNA ratio. In all reactions presented in this work the RNA/DNA ratio setted varied between 20 000 and 50 000. These small variations cannot explain the significant shift observed in the kinetics of hybridization where comparing the results with unincubated and incubated mixtures.

In our system, endogenous RNA polymerase seems to elongate RNA chains but very poorly reinitiate RNA transcription. Thus, initiation of RNA chains estimated by [γ-^{32}P]GMP incorporation into RNA is very low as compared to initiation by exogenous RNA polymerase (table 1). In order to measure the MMTV RNA synthesis under conditions of RNA chain initiation we have transcribed the same chromatin with

Table 1
In vitro synthesis of MMTV RNA

Experiment No.		RNA polymerase/ template ratio	Initiation with [γ-^{32}P]GTP cpm (incorporated into RNA)	Total RNA synthesis (μg/mg DNA)	Treatment of GR cells	MMTV RNA (%)
No incubation	I				− Dex	0.004
	II				+ Dex	0.002
Incubation −XTP						0.002
MMTV RNA						100
Incubation	I				− Dex	0.004 ± 0.001
(endogenous RNA	II		nd	nd	+ Dex	0.030
polymerase)	III		nd	nd		0.025 ± 0.003
	IV		330	2.5		0.030
Calf thymus	I	1.2			− Dex	0.010 ± 0.005
RNA polymerase B	II	1.2	3600	6	+ Dex	0.200
	III	1.2	nd	7		0.250 ± 0.02

This table summarizes results of several different transcription experiments with endogenous RNA polymerase and calf thymus RNA polymerase B (5 units). Chromatin isolated from GR mammary cells Dex treated for 6 h was transcribed as described in Materials and methods. [γ-^{32}P]GMP and [^{3}H]UMP incorporation into RNA were measured by precipitating an aliquot of the incubation mixture with 5% trichloroacetic acid. Acid-precipitated material was collected on glass filters (G. F. Whatman Co.). Filters were dried and radioactivity was counted in a Beckman liquid scintillation counter: [γ-^{32}P]GTP spec. radioact. 1600 cpm × pmol, [^{3}H]UTP spec. radioact. 60 cpm × pmol

purified calf thymus RNA polymerase B which synthesizes RNA relatively efficiently in vitro (table 1).

After measuring the amount of MMTV RNA synthesized by this enzyme (fig.1), we have found more MMTV RNA transcribed from chromatin with exogenous eukaryotic RNA polymerase B as compared to endogenous RNA polymerase (table 1). This result could be explained by a limiting amount of endogenous RNA polymerase molecules in relation to the 70 MMTV genomes integrated in GR mammary cells. Thus, exogenous calf thymus RNA polymerase B may partially saturate the chromatin template and transcribe (MMTV genomes) more efficiently.

3.2. *Hormonal regulation of MMTV gene expression*

Experiments with dexamethasone (Dex), a synthetic glucocorticoid show that GR cells treated with this hormone synthesize 20-fold more MMTV RNA [19]. To determine whether an in vivo dexamethasone treatment influences the in vitro transcription of MMTV genome we have compared the in vitro MMTV RNA synthesis using chromatin from cells treated with the hormone for 6 h and chromatin from untreated cells.

First, we have estimated the concentration of MMTV RNA in a cell-free system using endogenous RNA polymerase (fig.2). Unincubated chromatin from cells treated with Dex contains more MMTV RNA than chromatin from untreated cells. This MMTV RNA might represent higher amount of endogenous MMTV RNA. Furthermore, after incubation of the same chromatin for 1 h, about 30-fold more MMTV RNA can be detected than in the unincubated control. In contrast, using chromatin from untreated cells only about 10-fold MMTV RNA can be detected. With some reservation regarding the accuracy of this determination, the in vivo Dex treatment thus seems to increase about 3-fold the in vitro MMTV RNA synthesis by endogenous RNA polymerase. This stimulation of MMTV RNA synthesis could be due to an increase in its rate of synthesis, a decrease in its rate of degradation or a combination of the two mechanisms. In order to test the first of these possibilities we have measured the in vitro MMTV RNA synthesis by calf thymus RNA polymerase B, that is under conditions of RNA chain initiations. In fig.3 are shown annealing curves measuring MMTV RNA transcribed by eukaryotic enzyme from chromatin of untreated or hormone treated cells. After calculation of $crt_{1/2}$ and percent MMTV RNA it can be seen (table 1) that MMTV RNA synthesis is stimulated by a factor of 10, when chromatin from hormone treated cells is used.

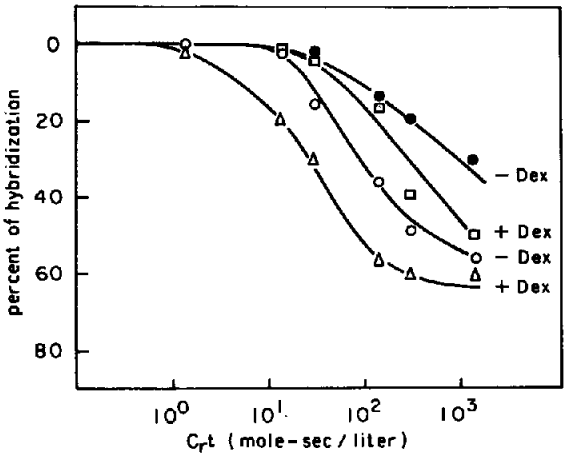

Fig.2. Dexamethasone effect on MMTV RNA synthesis with endogenous RNA polymerase. Chromatin was isolated from GR cells treated (□,△) or not (○,●) for 6 h with dexamethasone (10^{-6} M). The same amount of the two types of chromatin was unincubated (●,□) or incubated (○,△) for 1 h at 30°C.

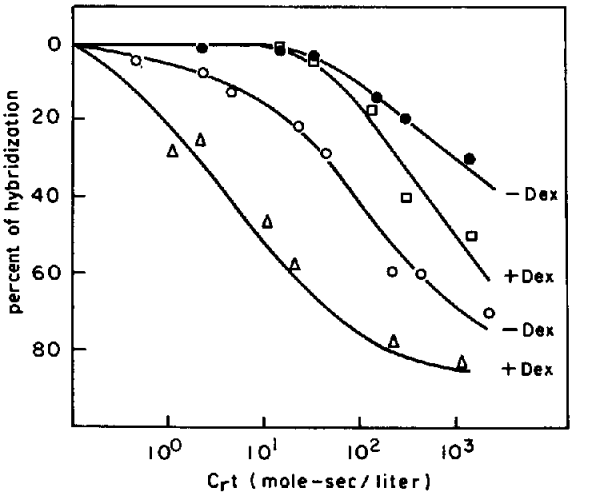

Fig.3. Effect of dexamethasone on the MMTV RNA synthesis by RNA polymerase B purified from calf thymus. Chromatin transcription was carried out under the same conditions as in fig.1 and fig.2. (●,□) RNA from unincubated chromatins. (○,△) RNA from chromatins incubated with 5 units of calf thymus RNA polymerase B.

Therefore, we can amplify the Dex effect on the in vitro MMTV synthesis by adding a higher amount of eukaryotic RNA polymerase B. This result strongly support the idea of a limiting amount of the RNA polymerase B present in this chromatin.

Finally, this 10-fold increase of MMTV RNA synthesis, close to the percent stimulation observed in vivo [19], provides very good support to the proposal that glucocorticoïds stimulate directly the MMTV RNA synthesis at the transcription level. Whereas these results strongly suggest that dexamethasone increases the rate of synthesis of MMTV RNA chains and/or the stability of MMTV RNA. Definitive evidence that Dex modifies only the rate of synthesis of MMTV RNA is dependent on the use of a chromatin transcription system completely reconstituted with a Dex receptor complex added in vitro.

Dedication and acknowledgments

To Gordon Tomkins – this work initiated in his laboratory is dedicated as a sign of my appreciation for an original scientist, gifted teacher and good friend.

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