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Effect of DNA supercoiling on in vitro transcription from the adenovirus early region 4

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The effect of DNA supercoiling on in vitro transcription from the early region 4 (E4) promoter of adenovirus type 5 (Ad5) has been investigated by using a cell-free transcription system that maintains supercoiled DNA templates. The supercoiled DNAs yield several-fold higher levels of E4 transcripts and more faithfully reflect the regulation of in vivo transcription than the linear DNAs.

Transcription; DNA supercoiling; Adenovirus early region 4; Deletion mutation; Transcription regulation; (Silk gland)

1. INTRODUCTION

Recent studies have indicated that a specific DNA topology is required for the expression of certain eukaryotic genes [1,2]. In chromatin, most DNase I-hypersensitive regions, which are regulatory regions of transcriptionally active genes, are sensitive to chemicals and S_1 nuclease, that attack altered DNA conformations [3,4]. This suggests that chromatin DNA in the DNase I-hypersensitive regions is in a superhelical state. Supercoiled DNA also yields a higher level of expression than linear DNA when introduced into living cells [5-7]. To investigate the possibility that torsional stress of DNA plays a direct role in the regulation of transcription, a cell-free transcription system that maintains superhelical DNA templates has been developed using a *Bombyx mori* silk gland extract [8].

Using this in vitro system, we have examined the effect of DNA topology on the regulation of transcription from the early region 4 (E4) of adenovirus type 5 (Ad 5).

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2. MATERIALS AND METHODS

2.1. *Plasmids*

Plasmid pSmaIL containing the Ad5 E4 wild-type (WT) promoter [9], and plasmids pAd500 [10] and pFLBH [9], containing the major late promoter (MLP), were used. Various deletion mutations in the E4 promoter region were constructed as described [11]. Plasmids pSmaIL and pAd500 DNAs were digested with *Tth*111I and *Pvu*II, respectively, for linearization.

2.2. *Preparation of cell extract and fractionation*

Preparation of a silk gland cell extract and its fractionation were carried out as described [8,10].

2.3. *In vitro transcription*

In vitro transcription reactions and analyses of RNA products were carried out as described [10].

3. RESULTS AND DISCUSSION

It has been demonstrated that both activities of relaxing supercoiled DNA and supercoiling relaxed DNA exist in a silk gland cell extract, suggesting that the topological state of DNA substrates in the extract is determined by the equilibrium between

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these activities [10]. Upon fractionation of the extract through a phosphocellulose column, supercoiling activity was reconstituted from two fractions eluting at 0.04 M and 0.6 M KCl [10]. To facilitate the analysis of supercoiling activity in these fractions, a closed circular form of pSmaIL DNA was catalyzed to adopt a relaxed form by using DNA topoisomerase I as described [8]. A relaxed form could be changed into a partially supercoiled form only on addition of a silk gland extract (fig.1, lane 5). On addition of both fractions to a silk gland extract, further supercoiling occurred (lanes 6,7). Linearized DNA, however, remained unaltered even on addition of these fractions (lanes 2,3). Using this system in which a supercoiled form was maintained during a reaction, the activities of transcription in vitro from the E4 promoter were investigated for different topological states. The supercoiled DNAs are several-fold more effective as templates than the linear DNAs (fig.2). A similar enhancement effect of supercoiling on transcription was observed not only for the E4 promoter but also for other promoters of Ad2 ML, silk fibroin and silk sericin genes [10]. Nevertheless, this phenomenon is not universal, since a number of other genes such as the *Drosophila* hsp70 gene did not respond to the supercoiling effect [10].

The relative transcription activities for various deletion mutations (fig.3) vs that of the wild type in different topological states were investigated by using a silk gland extract (fig.4 and table 1), the results being then compared with those obtained in vivo and in vitro on a HeLa cell extract described in [11]. In the linear state, the in vitro phenotypes of mutations obtained with silk gland extract are

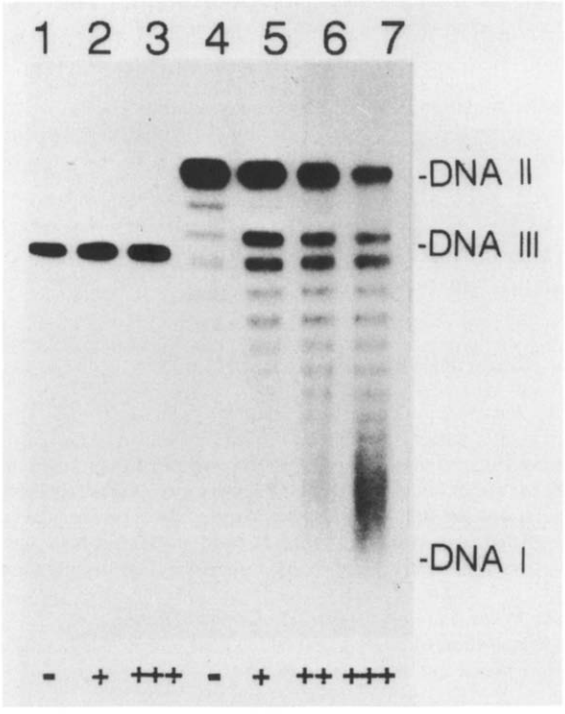

Fig.1. DNA supercoiling in silk gland extract supplemented with phosphocellulose fractions. Radioactive linear (lanes 1-3) or relaxed closed circular DNA (lanes 4-7) of pSmaIL (0.5 μg/ml) was incubated with silk gland extract (7.5 mg protein/ml) alone (lanes 2,5) or the same extract supplemented with 5 μg protein of the 0.04 M KCl eluate and 1 μg protein of the 0.6 M KCl eluate (lane 6) and with 10 μg protein of the 0.04 M KCl eluate and 2 μg protein of the 0.6 M KCl eluate (lanes 3,7). Mixture contained poly (dI-dC)·poly(dI-dC) (8 μg/ml) to increase bulk DNA concentrations. After incubation for 60 min at 30°C, DNA was extracted and analyzed by electrophoresis in a 1% agarose gel as in [8]. Positions of supercoiled, nicked circular and linear DNAs are denoted DNAI, DNAII and DNAIII, respectively.

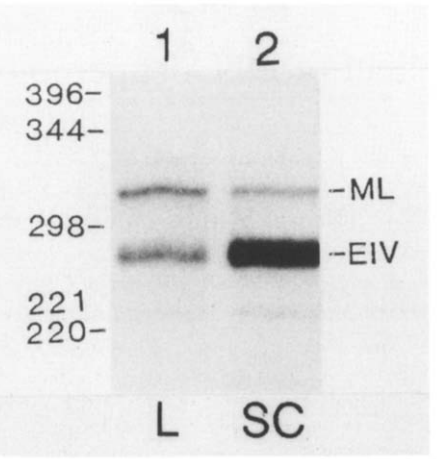

Fig.2. Effect of supercoiling on transcription from the EIV promoter. Linear (lane 1;L) or supercoiled (lane 2; SC) DNA of pSmaIL (24 μg/ml) was used as a template. A linearized template of pAd500 (6 μg/ml) was added to a reaction mixture (12.5 μl) as an internal control. In vitro transcripts were quantitated as in [10]. Radioactive transcripts were hybridized with M13 single-stranded recombinant (M13XE11 [9] and M13Ad4 [10]) DNA containing the coding strand of the E4 and ML gene, respectively. After treatment with S_1 nuclease, RNA/DNA hybrids were analyzed by electrophoresis on a neutral acrylamide gel. Transcripts from the E4 promoter and MLP were distinguished via the sizes of their S_1-resistant hybrids, 251 and 308 base pairs, respectively. ML and EIV show transcripts from ML and E4 promoters, respectively.

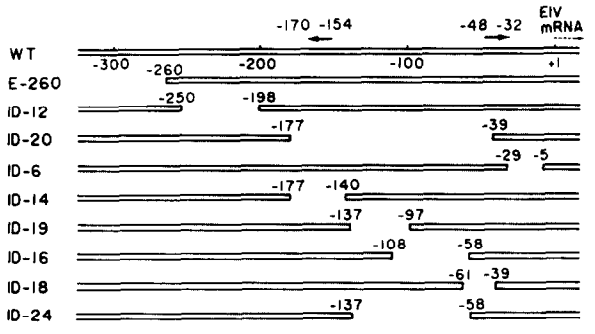

Fig.3. Structure of the various deletion mutations. The number of nucleotide residues from the furthest upstream cap site (+1) of E4 mRNA is shown. The endpoint of deletion for each mutation is shown as double lines for the E4 gene sequences. Straight arrows show positions of two homologous inverted repeats, the dashed arrow denoting transcripts from the E4 promoter.

consistent with those of the HeLa cell extract. As concerns ID-16, ID-19 and ID-24, lacking the sequences between the homologous inverted repeats, their transcription activities are lower than that of the wild type in the superhelical state. The relative activities correlated well with the in vivo results. These DNA templates in the linear state, however, revealed enhancement due to reasons unknown. We have identified two transcription factors, E4TF1 and E4TF3, in a HeLa cell extract and determined their binding sites in the E4 promoter [12]. Two of the four E4TF3-binding sites were present in both of the inverted repeats, located at around -40 and -160, and the E4TF1-binding site was located at around -140, close to the distal E4TF3-binding site. Similar binding activities were noted in a silk gland extract via gel electrophoresis DNA-binding assays (not shown). Since the ID-16, ID-19 and ID-24 mutations lack the sequences between the binding sites of these factors and the binding sites are relocalized to positions closer to each other, it is possible that the interaction of these transcription factors may alter for different topological states.

The phenotype of the ID-20 mutation in the supercoiled state was also similar with respect to the in vivo results (table 1). It indicated that the region encompassing both of the inverted repeats

Table 1

Comparison of relative transcription activities of the E4 mutant promoters in different assays

	Transcription in vitro			Transcription in vivo	
	Supercoiled circular DNA (p.s.g.)	Linear DNA (p.s.g.)	Linear DNA[a] (HeLa)	Colony[a] formation	CAT[a] activity
WT	100	100	100	100	100
E-260	66	86	70	75	79
ID-12	65	85	63	47	89
ID-20	17	65	131	19	18
ID-6	0	0	0	40	87
ID-14	43	32	25	41	57
ID-19	50	195	110	35	40
ID-16	82	131	200	24	65
ID-18	22	23	75	42	74
ID-24	64	170	135	N.D.	39

[a] Data (except for ID-24) were taken from [11]. In vivo activity of mutant promoters was determined by assaying expression of the reporter genes after introduction of the supercoiled circular plasmid DNA into recipient cells. p.s.g., posterior silk gland extract; HeLa, HeLa cell extract; N.D., not done

The intensity of bands in fig.4 was quantitated by tracing the autoradiographs on a densitometer (ATAGO). The amounts of the mutant bands were calculated relative to that of pFLBH (internal control in the reaction mixture). The percentage of transcriptional activity of each mutant vs that of the wild type (pSmaIL) was calculated and listed

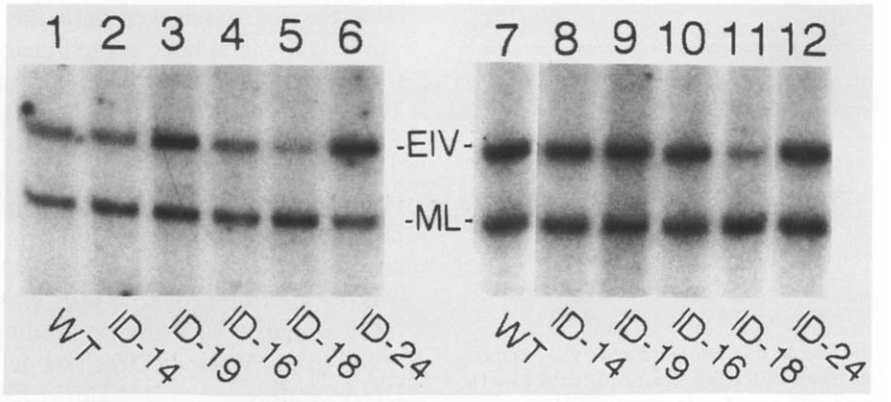

Fig.4. Relative activities of in vitro transcription of various mutations to that of the WT in different topological forms. The in vitro transcription reaction was performed as in [10]. A mixture of plasmid DNA of each mutation (20 μg/ml) in the linear (lanes 1–6) or supercoiled (lanes 7–12) form and *Pst*I-cleaved pFLBH DNA (10 μg/ml) used as an internal control was added to the reaction mixture. Transcripts from the E4 promoter and MLP were hybridized with M13XE11 [9] and M13XH11 [9] DNAs, thereafter being treated with S_1 nuclease and hybrids analyzed as described above. Transcripts from E4 and MLP were distinguished via the size of their S_1-resistant hybrids (251 and 197 base pairs, respectively). ML and EIV represent the transcripts from the ML and E4 promoters, respectively.

was a major domain for efficient transcription in vitro in the supercoiled but not in the linear state. It is suggested that deletion of the domain alters the interaction of the factors involved in transcription from the mutant promoter in the different topological states.

For other mutations, except ID-6, supercoiled templates showed almost the same phenotypes as the linear forms of the corresponding mutations. These show a good corelation with previous in vivo results [11]. Irrespective of template topology, deletion of the TATA box (ID-6) results in less efficient transcription in the in vitro systems for both silk gland and HeLa cell extracts. Transcription in vivo from the E4 promoter is not as strongly dependent on the TATA box as that in vitro [11], suggesting that the dependency of the initiation of transcription from the E4 promoter on the TATA box is specific for an in vitro system. The in vivo results might arise from transcripts initiating at heterogeneous sites due to deletion of the TATA box as described [13–15].

By utilizing the cell-free assay described here, it should be possible to analyze more faithful regulation of transcription in vitro from the E4 promoter through a variety of viral [16–20] and cellular [14,21] trans-acting factors.

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