Initiation of Transcription in Normal and Phosphorylated Rat Liver Nucleosomes

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Initiation sites were enumerated in rat liver nucleosomes with *Escherichia coli* RNA polymerase. One site was present in approx. 3.5 mononucleosomes and in a 1200-base-pair length of polynucleosomes. S-phase nuclei or normal nuclei phosphorylated *in vitro* showed increased numbers of sites; the elongation rates were unchanged.

Bustin (1978) showed that transcription on liver nucleosomes, as with intact chromatin, is restricted as compared with free DNA. Micrococcal-nuclease digestion of rat liver nuclei selectively releases mononucleosomes associated with phosphorylated histone H1, and in S-phase with new DNA (Ord & Stocken, 1978). The transcriptional properties of nucleosomes and core particles (140-base-pair DNA) released from normal liver, from nuclei after phosphorylation *in vitro*, and from nuclei released from partially hepatectomized livers in S-phase, were analysed to distinguish initiation sites for RNA polymerase and rates of elongation (Cedar & Felsenfeld, 1973). Initiating sites were especially associated with interbody regions of the nucleosomes. The number of sites was increased by phosphorylation in all classes of nucleosomes, and also after partial hepatectomy. Elongation rates on mononucleosomes were not altered by these procedures and were three times faster than for core particles.

Experimental

Methods

Liver nucleosomes were prepared from male rats of 350 g body wt. and analysed as described elsewhere (Ord & Stocken, 1978). Rats (12 sham-operated and 20–24 partially hepatectomized) were killed 22 h after operation, a time when the peak rate of DNA synthesis occurs in the experimental animals. Two preparations of nucleosomes were available from partially hepatectomized rats, three from control nuclei that had been phosphorylated *in vitro* with [γ - 32 P]ATP and protein kinase (EC 2.7.1.37) (Ord & Stocken, 1978), and six control preparations. Mononucleosomes contained 200-base-pair DNA, histone H1 and core histones, core particles, 140-base-pair DNA and core histones. Pooled mononucleosomes removed from sucrose gradients frequently contained both 200- and 140-base-pair DNA particles. These gave results that were intermediate

between those of the two constituents, and are not included in the results. Phosphorylation *in vitro* yielded nucleosomes that were diminishingly phosphorylated from mononucleosomes to polynucleosomes; histone H1 was the histone principally phosphorylated. Nucleosomes obtained from liver in S-phase contained new DNA, phosphorylated histone H1 and core histones. Phosphorylation again diminished from mononucleosomes to trinucleosomes (Ord & Stocken, 1978).

Assays with RNA polymerase (EC 2.7.7.6) were performed as described by Bustin (1978), who used procedures developed by Cedar & Felsenfeld (1973). Initiation sites were determined by preincubating DNA or nucleosomes for 20 min at 37°C with ATP, GTP, Mn²⁺ and enzymes. (NH₄)₂SO₄ was then added to 0.4 M, followed by CTP and 5-[3 H]UTP (sp. activity 10 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). Transcription in high salt was for 15 min at 37°C. The reactions were stopped by the addition of trichloroacetic acid to 7% (w/v), the samples filtered through Whatman GF/C glass-microfibre papers, and washed with 10% trichloroacetic acid and ether. Normally 1 unit of *Escherichia coli* RNA polymerase type II [about 700 units/mg; Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] was used per assay. In some experiments, initiation was stopped by rifampicin (Sigma) (Bustin, 1978) before the addition of CTP and UTP. When transcription was to be monitored continuously in low salt, all the reactants were present before the addition of the enzyme; the reaction then proceeded for 10–30 min at 37°C. All assays were in triplicate, and appropriate procedures with calf thymus DNA (type I, highly polymerized; Sigma) were included in each experiment. Controls without template gave <5% of the experimental radioactivity, and no endogenous RNA polymerase could be detected in the nucleosomes. The specific radioactivity of [3 H]UTP in the assays was about 1500 c.p.m./pmol. No turbidity or precipitation was present in the incubation mixtures.

Results and Discussion

Preliminary results with transcription in low salt suggested that rates of transcription of mononucleosomes through trinucleosomes were inversely related to the size of the nucleosome; with core particles containing 140-base-pair DNA, transcription was markedly decreased (Fig. 1).

Differences in transcription may be due to differences in the number of initiation sites, the elongation rate (Cedar & Felsenfeld, 1973) and, with short lengths of DNA, the length of template. To analyse these parameters, initiation sites were determined by titrating *E. coli* RNA polymerase with DNA and nucleosomes (Fig. 2). One unit of RNA polymerase was saturated by 0.30–0.35 μ g of DNA, a result identical with that reported by Bustin (1978) who calculated, with enzyme of nearly the same specific activity as ours, that one molecule of enzyme was bound per 185 base-pairs of DNA. Amounts of nucleosomal DNA saturating 1 unit of RNA

Fig. 1. Transcription of *G*₀-phase liver nucleosomes by *E. coli* polymerase

Nucleosomes containing 5 μ g of DNA were present for each assay, with 2 units of enzyme. Transcription was monitored by measuring [^3H]UTP uptake for 15 min at 37°C in low salt (see the text). ---, Incorporation with 5 μ g of DNA.

Fig. 2. Determination of initiation sites by the high-salt technique

For details, see the text. RNA polymerase (1 unit) was present for all the assays. O, Calf thymus DNA; ●, dinucleosomes from liver nuclei in *G*₀-phase that had been phosphorylated *in vitro*; Δ, core particles from liver nuclei in *G*₀-phase.

polymerase followed curves that were asymptotic with respect to both axes (Fig. 3). Nucleosomes of approx. 1200-base-pair DNA bound 1 unit of enzyme (Fig. 3); this is about 15% of the number of sites for *E. coli* polymerase in calf thymus DNA. With mononucleosomes, 700 base-pairs were required/unit enzyme, i.e. 1 in 3.5 mononucleosomes bound enzyme. Differences in initiation sites between mononucleosomes and trinucleosomes were confirmed by using rifampicin. With this technique the ratio (no. of initiation sites on control trinucleosomes)/(no. on mononucleosomes) was 1.67:1, compared with 1.86:1 found by the $(\text{NH}_4)_2\text{SO}_4$ procedure.

The number of sites/unit length of nucleosome declined sharply between mononucleosomes containing 200-base-pair DNA and histone H1, and core

Fig. 3. Initiation-site assays for liver nucleosomes from liver nuclei in G_0 -phase, from partially hepatectomized rats 22h after operation and from G_0 -phase liver nuclei that had been phosphorylated *in vitro*

Initiation sites are expressed as the amount of DNA (μg) required to saturate 1 unit of RNA polymerase. ●, Control nuclei, G_0 -phase; ○, control nuclei phosphorylated *in vitro*; ▲, nuclei from partially hepatectomized rats.

particles with 140-base-pair DNA (Fig. 3). The interbody region of the nucleosome, therefore, rather than the core, carried initiation sites, which was confirmed when mononucleosomes were used that had been prepared in gradients containing 0.6M-NaCl. 0.6M-NaCl strips histone H1 and some non-histone proteins from the mononucleosomes. The protein-depleted mononucleosomes titrated at 0.55 μg of DNA, that is polymerase bound to about 50% stripped mononucleosomes as compared to 28% of the control mononucleosomes.

The saturation value for transcription achieved when initiation sites are determined (Fig. 2) can also give information about elongation in high salt (Cedar & Felsenfeld, 1973). Mononucleosomes through to pentanucleosomes showed the same plateau as did DNA, but with core particles only 74% of this value was reached (Fig. 2). Bustin (1978) found the length of RNA transcribed with liver nucleosomes

to be 150 nucleotides; our data are consistent with a length greater than 140 base-pairs and less than 200.

We have also measured continuous transcription in low salt with excess polymerase (three times the amount required to saturate the initiation sites). Under these conditions, reinitiation can occur. Rates with mononucleosomes were 46% of those with DNA (Fig. 4). If the rate of binding of the enzyme to initiation sites does not differ between DNA and nucleosomes, decreased transcription under these conditions indicates decreased rates of elongation. This was reported previously for mononucleosomes (Bustin, 1978) and chromatin (Cedar & Felsenfeld, 1973). With core particles the rate was only 15% of that with DNA (Fig. 4), indicating that the rate of elongation through the region where DNA is bound to the histone core is markedly diminished, and suggesting that the rate through the interbody region is approximately that for free DNA.

Fig. 4. Transcription in low salt with high enzyme/initiation-site ratios

RNA polymerase (0.1 unit) and 0.5 μg of DNA from G_0 -phase mononucleosomes and core particles, or 0.1 μg of calf thymus DNA, were present in the reaction mixture. ●, DNA, ○, mononucleosomes; ▲, core particles.

Initiation sites were also determined for nucleosomes from G₀-phase liver nuclei that had been phosphorylated *in vitro*, histone H1 being the histone chiefly affected, and from partially hepatectomized livers in S-phase (Fig. 3). The curves were of the same shape as for the controls; phosphorylation increased the number of sites per mononucleosome by 25% and per trinucleosome by 50%. In S-phase, the sites per monosome were 73% increased over G₀-phase values, and for trinucleosomes the number of sites was doubled. In low salt, with high enzyme/DNA ratios, the same rates of transcription for G₀- and S-phase monosomes were found, indicating identical rates of elongation.

The procedure used for digestion with micrococcal-nuclease releases from liver nuclei, in G₀-phase, mononucleosomes associated with ADP-ribosylated histone H1, and in S-phase, mononucleosomes associated with new DNA, and phosphorylated histone H1 and core histones (Ord & Stocken, 1978). RNA synthesis increases in partially hepatectomized livers; this is reflected in the increased numbers of initiation sites found in nucleosomes from S-phase nuclei (Fig. 3).

The present results suggest that the greater phosphorylation of the histones in control mononucleosomes compared with polynucleosomes (Ord & Stocken, 1978) may explain their relatively larger proportion of initiation sites. Phosphorylation *in vitro* gave more sites on all the released nucleosomes.

Interpretation of the results, however, depends critically on the significance of transcriptional studies with *E. coli* polymerase and eukaryotic chromatin. Gjerset & McCarthy (1977) found that transcription of satellite DNA was decreased compared with other regions of mouse chromatin, and that aberrant transcription was further diminished with low (1:10) enzyme/DNA ratios, conditions very different from those required here in the initiation studies.

Results with 140-base-pair DNA particles strongly indicate that restrictions in chromatin to initiation and propagation of RNA synthesis are particularly associated with the nucleosome-core region. This restriction may be partially released by ADP-ribosylation of histone H3 and phosphorylation of histone 2A, events that can occur where histone H1 has been phosphorylated (Ord & Stocken, 1978).

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