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In vitro transcription with extracts of nuclei of *Drosophila* embryos

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A B S T R A C T

An in vitro transcription system has been developed from 0.3M NaCl extracts of nuclei of Drosophila embryos. Optimal transcription in the Drosophila embryo extract (DEX) was at 5mM $MgCl_2$, 70mM KCl, 25°C and with promoter concentrations of 0,75-1.0 pmol/assay. In vitro transcription from the Adenovirus-2 major late and the Drosophila histone gene promoters was studied in particular. S1-nuclease protection experiments showed that in vitro transcription from these promoters was accurate. In vitro transcription from the Adenovirus-2 major late promoter was less efficient than from histone gene H3 and H4 promoters in DEX. Vicecersa, in vitro transcription from Adenovirus-2 major late promoter was more efficient in HeLa whole cell extracts. The efficiencies of transcription from histone gene promoters decereased in DEX in the order H4>H3>H2a. Transcription from H2b and H1 promoters was not detected in DEX. The transcription from the Adenovirus-2 major late promoter was completely inhibited by histone H3 and H4 promoters. Preincubation of DEX with the adenoviral template, however, did not inhibit transcription from histone H3 and H4 promoters. The transcription start sites of histone genes H3 and H4 are separated by 160 base pairs. The H3 and H4 transcription start sites were subcloned separately. Now, a competition of transcription from the H3/H4 promoters with the Adenovirus-2 major late promoter was observed. The competition studies suggest that preincubation of DEX with the adenoviral template inhibited transcription from the H3 promoter more strongly than from the H4 promoter.

I N T R O D U C T I O N

The development of in vitro transcription systems has significantly contributed to an understanding of the molecular events underlying the initiation of transcription (see 1,2 for reviews). In vivo studies have shown that the initiation of transcription is controlled by several control elements which are upstream or downstream of the transcription start site (see 3 for review and references). The in vivo function of these regulatory elements has been reproduced in vitro (see 1,2,4 for reviews).

Upstream polymerase B promoter elements may be classified into three types based on their function, their sequence characteristics and their distance relative to the start site of transcription. The TATA-box element is located 25 to 30 bp upstream from the transcription start site. This element is important for specific transcription (i.e. for fixing the in vivo start site(s). The second type of element is located 40 to 110 bp upstream from the transcription start site, frequently referred to as CCAAT-box element. These elements are apparently important for the efficiency of transcription, i.e. the amount of transcripts produced per transcription unit. The third type of element can stimulate transcription from considerable distances independent of orientation relative to the transcription start site. These elements are referred to as enhancer elements. They are thought to be tissue-specific modulators, which convey, for instance, hormonal, stimuli (5).

In vitro transcription from a number of viral and cellular genes, such as the Adenovirus -2 major late (Adomal)(6,7), SV 40 early (8-10), sea urchin histone H2a (11), Drosophila histone H3/H4 (12) and Drosophila heat shock gene (hsp 70) (13) transcription units, required multiple complementary factors for specific and efficient transcritpion by RNA polymerase B. These recent competition and footprinting experiments have shown that the various upstream control elements are apparently recognized by transcription factors, which bind either to promoter regions of many genes (TATA-box element binding proteins) or bind specifically to upstream sequences of particular genes such as SP1-protein to the SV40 enhancer element (10) or HSTF protein to the hsp 70 transcription unit (13,14). Since upstream sequences of many genes are not conserved across species, species specific proteins may regulate the efficiency of transcription initiation from polymerase B promoters. The activities of these proteins in vitro could possibly be detected in a homologous in vitro transcription system. (15)

Many Drosophila genes are now available in isolated form and are amenable to studying the regulation of transcription in vitro as well as in vivo. Therefore, we have developed a simple procedure for preparing a Drosophila in vitro transcription system. The source of this system are nuclei of Drosophila embryos, which can readily be isolated in sufficient quantities by any Drosophila laboratory. In this paper we describe the general properties of this Drosophila transcription system as well as the transcrip-

tion from the Adomal- and the Drosophila histone H1, H2a/H2b, H3/H4 transcription units.

MATERIALS AND METHODS

Extract preparation. Freshly collected and washed 0-12 hr embryos were resuspended in 10 mM Tris-HCL (pH 7,5), 1,5 mM $MgCl_2$, 0,3 mM $CaCl_2$, 0,5 mM dithiothreitol, 0,1 mM leupeptin, 2 % (w/v) dextrane 171000 buffer (Buffer A) at a concentration of 1 g wet weight/ml. They were homogenised in a glass-teflon-homogeniser (Braun-Melsungen, FRG) with 5 strokes at 150 rpm and with 5 strokes at 200 rpm. The resultant slurry was filtered through 3 layers of miracloth. The filtrate was diluted sixfold. Nuclei were sedimented in a table-top centrifuge. They were resuspended in 1,5 times the original volume and were recentrifuged at 20-000g for 20'. The sediment was taken up in 0,5 ml buffer B/g sediment. Buffer B was 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (Hepes) KOH (pH 8.0), 2,5 mM $MgCl_2$ 0,5 mM $CaCl_2$, 0,5 mM dithiothreitol, 20 % glycerol, 0,1 mM leupeptin. Another 0,5 ml 1,2 M NaCl containing buffer B /g sediment was added dropwise. Debris was removed by high speed centrifugation (30', 40.000 rpm, SW60 rotor). The lipid layer was taken off. The remaining supernatant was dialysed three times for 1,5 hr against 8,5 mM $MgCl_2$, 120 mM KCl, 0,5 mM dithiotheirol, 20 % glycerol, 10 mM Hepes-KOH (pH 8.0) buffer. The supernatant was cleared again (30', 30.000 rpm, SW60 rotor). The Drosophila extract (DEX) was stored at-70°C until use. The extract was stable for more than 2 months under these conditions. All procedures were carried out at 4 °C.

In vitro transcription

Transcription assays were carried out in a final volume of 25 µl 10 mM Hepes-KOH (pH 8,0), 5 mM $MgCl_2$, 70 mM KCl, 0,5 mM dithiothreitol, 3 mM creatin phosphate, 1 mM ATP, GTP, CTP and UTP. 15 µl DEX were used for each assay. DNA- and promoter concentrations are given in legends to the figures. Reactions were terminated by the addition of 10 mM vanadyl-inhibitor and 15 µg DNAse I. Incubation was continued for 15' at 30 °C. Lauroylsarkosine was added to a concentration of 0,6 %. Protein was digested with 10 µg proteinase K for 30 ' at 37°C. The volume of the assay mixture was increased to 200 µl with 10 µg/ml tRNA and 50 mM Na acetate (pH 4,5), 0,6 % lauroylsarkosine, 0,15 M NaCl buffer. After phenol/chloroform extraction RNA was precipitated with 2,5 vol. ethanol. The RNA pellet was processed

together with the 5'- endlabelled DNA-fragment for hybridization and S1-nuclease digestion (16) PAGE of S1-nuclease protected DNA-fragments was as described by Maxam and Gilbert (17). Dried gels were autoradiographed at -70°C with an intensifier screen (Cronex, Du Pont). Radioactivity in dried gels was measured by determining Cerenkov-radiation of cut out bands. Background radiation was 5 % in gel pieces above and below the respective radioactive band.

RNA Polymerase B activity in DEX was measured by adding 15µl DEX to a final volume of 200 µl 10mM Tris-HCl (pH7.5), 2.5mM $MgCl_2$, 2mM $MnCl_2$, 0.1mM EDTA, 200mM $(NH_4)_2SO_4$, 1mM ATP, GTP, CTP, 0,025mM UTP, 2µCi ^{3}H-UTP (42Ci/mmol), 250µg salmon-sperm DNA/ml. The reaction mixture was incubated at 25°C for 30'. RNA was precipitated with trichloro acetic acid. Precipitates were collected on Whatman GFC-filters. Washed filters were transferred into scintillation vials and ^{3}H-counts of ^{3}H-UTP incorporated into RNA were measured in a Beckman LS 9000 scintillation counter. In paralell experiments, 8µg α-amanatin/ml reaction mixture was added. RNA Polymerase B activity was calculated from the difference obtained from the incorporation of ^{3}H-UTP into RNA in the presence or absence of α-amanitin. All points in Figure 2C were done in duplicate.

A HeLa-whole cell extract (WCE) was prepared according to Manley et al. (18). Preparation of plasmids, restriction digests, DNA-fragment-isolation, subcloning and endlabelling followed the procedures given by Maniatis et al. (19). DNA-fragments were sequenced as described by Maxam and Gilbert (17).

Construction of Recombinants. Recombinants were constructed using standard cloning techniques (19). Briefly, plasmid cDM 500 (a generous gift of D. Hogness, Stanford University) was digested with HaeII. Overhanging 3'-ends were removed with T4 DNA-polymerase. The fragments were ligated with HindIII linkers and cloned into the HindIII site of pAT153 vector. This yielded the three subclones pATH1, pATH2a/b, pATH3/4 (Fig. 1). The subclones contained 3'-deleted histone genes and upstream sequences as indicated. pAT△H3 and pAT△H4 were constructed from pATH3/4 in the following way. Subclone pATH3/4 was restricted with AvaI, which gives a 2.5 kb- and a 1,55 kb-fragment. The 2,5 kb-fragment was religated to yield pAT△H3. The 1,55 kb-fragment was ligated into the AvaI site of pAT153 to yield pAT△H4 (Fig. 1). Recombinant pWAB was constructed by recombining the 1,1 kb XhoI-BglI-fragment of cDM 500 with the 2,6 kb BamH1-AvaI frag-

ment of pAT153. The one base-pair mismatch between the AvaI and XhoI restriction site was repaired after ligation and transformation to a XhoI-site as shown in Fig. 1. Plasmid pAdv, containing the SmaF-fragment of adenovirus-2 was kindly provided by W. Keller (DKFZ, Heidelberg). This plasmid was restricted with SmaI. The 2,1kb-SmaI-fragment was extended with HindIII-linkers and was subcloned into pAT153 vector to obtain subclone pAdomal (Fig. 1). Each recombinant was checked by sequence analysis. Recombinant DNA was propagated in ER1 host-vector system under L2/B1 containment conditions, as defined in the guidelines of the Federal German Government for recombinant DNA research.
M-aminophenylboronate-Sepharose-column-chromatography was carried out as described (20, 21). Columns (60 µl bed volume) were saturated with 100 µg tRNA before use in order to minimize unspecific RNA-binding. RNA was eluted with 50 mM Na acetate (pH5,0), 200 mM NaCl, 0,1 % sodium dodecyl sulfate buffer. The cap site of RNA was digested with Tobacco acid pyrophosphatase as described (22).

RESULTS

Drosophila embryos were collected from Drosophila mass cultures. Crude nuclei were prepared by homogenising freshly collected embryos as described in Materials and Methods. These nuclei have been extracted with various sodium chloride concentrations in order to obtain a nuclear extract for *in vitro* transcription similar to recently described procedures (12, 23). The DEX extract contained DNA-exonuclease activities, which could not easily be removed or be inactivated (data are not shown). Therefore, we could not use linear DNA-templates for efficient synthesis of run- off transcripts. Instead, we have used circular DNA-templates in most experiments and have analysed the synthesis of specific transcripts by S1 - nuclease protection experiments (16). At first, the general transcription properties of DEX were investigated *in vitro* with recombinant pAdomal as template. pAdomal contains the major late Adenovirus-2 promoter (Fig.1). *In vitro* transcription with DEX was compared with the well characterized HeLa-WCE which accurately and efficiently initiated transcription at the start site of the Adenovirus-2 major late promoter (6,7). Drosophila nuclei were extracted with 0,1 to 0,5 M NaCl concentrations. The relative efficiency of specific pAdomal transcription (Fig. 2A, B) was determined in the various nuclear extracts. We have also determined unspecific α-amanitin sensi-

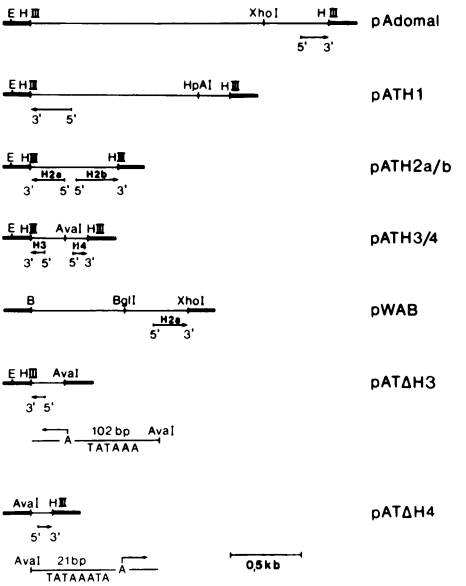

Fig. 1 DNA templates used in the transcription and S1-nuclease protection assays. The construction of the recombinants is described in Materials and Methods. The bold line indicates pAT-sequences. Arrows indicate *in vivo* start sites and direction of transcription. Transcript sizes are for the 3'- deleted adenovirus-2 major late transcription unit 197 nucleotides, the 3'-deleted Drosphila histone H1, H2a, H2b, H3 and H4 genes 285, 240, 290, 100 and 105 nucleotides, respectively. The endlabelled BglI-XhoI-fragment of recombinant pWAB was used for mapping the *in vivo* start site of H2a mRNA. Underneath pATΔH3 and pATΔH4, respectively, the distance of the TATA-box element to the AvaI-cloning site is given by the number of base pairs.

tive RNA synthesis in the various extracts with denatured salmon sperm DNA as template (Fig. 2C). These assays were used to estimate the relative amounts of active polymerase B in DEX, since we wanted to optimize our Drosphila *in vitro* transcription system under conditions, where the polymerase B concentration was not limiting the system. A comparison of the data in Figure 2B and 2C showed that nuclear extracts, which were prepared with increasing salt concentrations continually increased the efficiency of specific pAdomal transcription. The RNA-polymerase B concentration, on the other hand, reached a plateau at 0,3 M NaCl (Fig. 3C). Drosophila nuclei became very fragile at salt concentrations of 0,4 M NaCl and completely lysed at 0,5 M NaCl under our extraction conditions, making it difficult to prepare DEX with reprod-

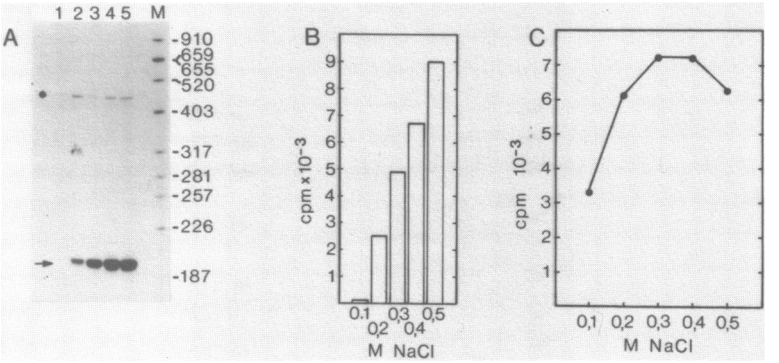

Fig. 2 in vitro transcription with Drosophila extracts prepared from nuclei at different salt concentrations. A. PAGE of S1-nuclease protected pAdomal DNA. The asterix indicates the size of the original 5' endlabelled XhoI-HindIII fragment, (specific activity 2 x 10^6 cpm/pmol 5'-end), the arrow indicates the 197 nucleotide fragment protected by pAdomal transcript. Nuclei of Drosophila embryos were extracted with 0,1M NaCl (Lane 1), 0,2 M NaCl (lane 2), 0,3 M NaCl (lane 3), 0,4 M NaCl (lane 4), 0,5 M NaCl (lane 5) containing buffer B as described in Materials and Methods. Lane M are 5'-endlabelled DNA-size markers. Exposure time of the gel was 1,5 hr. B. ^{32}P-counts in the 197 nucleotide long protected fragment in A were determined by cutting out the bands from the dried gel and by subsequently counting Cerenkov radiation. C. Polymerase B activity in the extracts prepared at the various NaCl concentrations. (see Materials and Methods). Denatured salmon sperm DNA was used as template. Polymerase B - dependent incorporation of ^{3}H-UTP into RNA was calculated from the difference in trichloro acetic acid precipitable material obtained by incubating without or with 8 µg/ml α-amanitin.

ucible properties. Therefore, we have used in all further experiments 0,3 M nuclear extracts, where the concentration of RNA-polymerase B was apparently not limiting for the activity of *in vitro* transcription.

Addition of 0,01 µg α-amanitin/ml reaction mixture reduced specific transcript synthesis by 40 %. This α-amanatin sensitivity is similar to that of unspecific transcription by purified Drosophila RNA-polymerase B (24). Specific RNA-synthesis was also inhibited by adding actinomycin D or heparin to the *in vitro* transcription assay (Fig. 3, lanes 7 and 9). These data indicated that pAdomal transcription was dependent on DNA-template as well as on active RNA-polymerase B.

The DEX-transcription system was optimized with respect to salt-concentration, DNA-concentration, incubation time and temperature. The optimal $MgCl_2$ and KCl concentrations for transcription

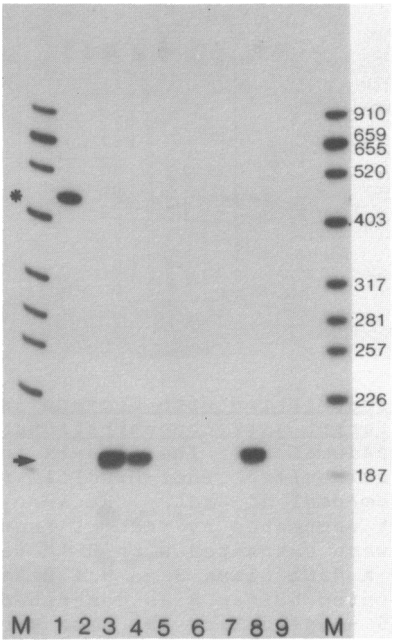

Fig. 3 Inhibition of in vitro transcription from Adenovirus-2 major late promoter by various drugs. *in vitro* transcription from pAdomal was assayed by S1-nuclease protection experiments (16) with a 5'- endlabelled XhoI-HindIII fragment (asterix, lane 1) (specific activity 1,5 x 10^6 cpm/pmol 5'-end). Lane 2 shows a control transcription from vector pAT153. Lanes 3, 4, 5, 6, are transcriptions from pAdomal with 0, 0,01, 0,1, and 8 µg/ml α-amanitin, lane 7 with 20 µg/ml actinomycin D and lanes 8, 9 with 1 µg/ml and 1mg/ml heparin, respectively. Lanes M are DNA size markers as indicated. The arrow indicates the 197 nucleotide long DNA-fragment protected by pAdomal transcript against S1-nuclease digestion. Exposure time of the gel was 3 hr. Cerenkow-counts/min in lanes 2 to 9 were 0, 5600, 3000, 400, 0, 0, 4200, and 0, respectively.

were at 5 mM and at 70 mM, respectively. (Fig. 4 A and B). Interestingly, purified Drosophila RNA-polymerase B is almost completely inactive under these salt concentrations (24). The Mg^{2+}- optima of accurate *in vitro* transcription were significantly higher for HeLa-nuclear extracts. Transcription from the Adenovirus-2 major late promoter was optimal at 10-12 mM Mg^{2+}, that from other DNA-templates (e.g. human histone H4, mouse ß-globin) at 8-10 mM Mg^{2+} (23).

The temperature optimum of Drosophila *in vitro* transcription was at 25°C (Fig. 4C). This is incidentally the optimum temperature

for culturing flies as well as Drosophila tissue culture cells (25). In contrast, HeLa-transcription systems have a temperature optimum at 30°C (1,4,18,23). The inactivation of the Drosophila transcription system at higher temperatures might be due to the degradation of DNA-template or of RNA-transcript. We have not observed a significant degradation of DNA or of RNA in DEX at higher temperatures (data not shown). As Drosophila RNA polymerase B itself is still 50 % active at 37°C (24), the complete inactivation of specific transcription at 37°C apparently reflects the heat-lability of factor(s) involved in transcription initiation (13).

The amount of Adenovirus-2 transcript synthesized by DEX increased for up to 90' (Fig. 4D). Incubation times of more than 90' lead to a rapid decrease in the number of stable RNA-transcripts. We have not analyzed the reasons for this decrease. α-amanitin was added to the in vitro transcription assay after 30' (Fig. 4D, dashed line). This immediately resulted in a dramatic reduction of RNA transcripts synthesized in the 30 ' following the addition of α-amanitin. This result might indicate that initiation of transcription still occurs in DEX after 30'.

DNA-template concentrations were optimal at 80-100µg pAdomal/ml transcription assay (Fig. 4F). This concentration corresponds to 1pmol promoter/ assay. It is relatively high, since other in vitro transcription systems require 2 to 5 times less DNA (1,4,12,18,23). 0,75 to 1pmol promoter/ transcription assay was also required for optimal in vitro transcription with DEX, if Drosophila histone genes were employed as DNA-templates. We have determined the number of RNA transcripts per transcription assay measuring the radioactivity of the endlabelled DNA-fragment, which remained after S1-nuclease digestion. As discussed below, the minimum transcription efficiency in DEX was 0,01 pAdomal transcript /gene/hour. This efficiency compares well with that in WCE-extracts (18,23).

After we had optimized the general conditions for in vitro transcription in DEX, in vitro transcription from Drosophila histone genes was investigated. For this purpose, we have subcloned the five Drosophila histone genes, as described in Materials and Methods. Drosophila histones H3/H4 and H2a/H2b, respectively, are transcribed in vivo in opposite directions (26). These histone gene pairs are apparently separated by common intergenic spacer-sequences. Therefore, we have cloned into pAT153 3'- truncated

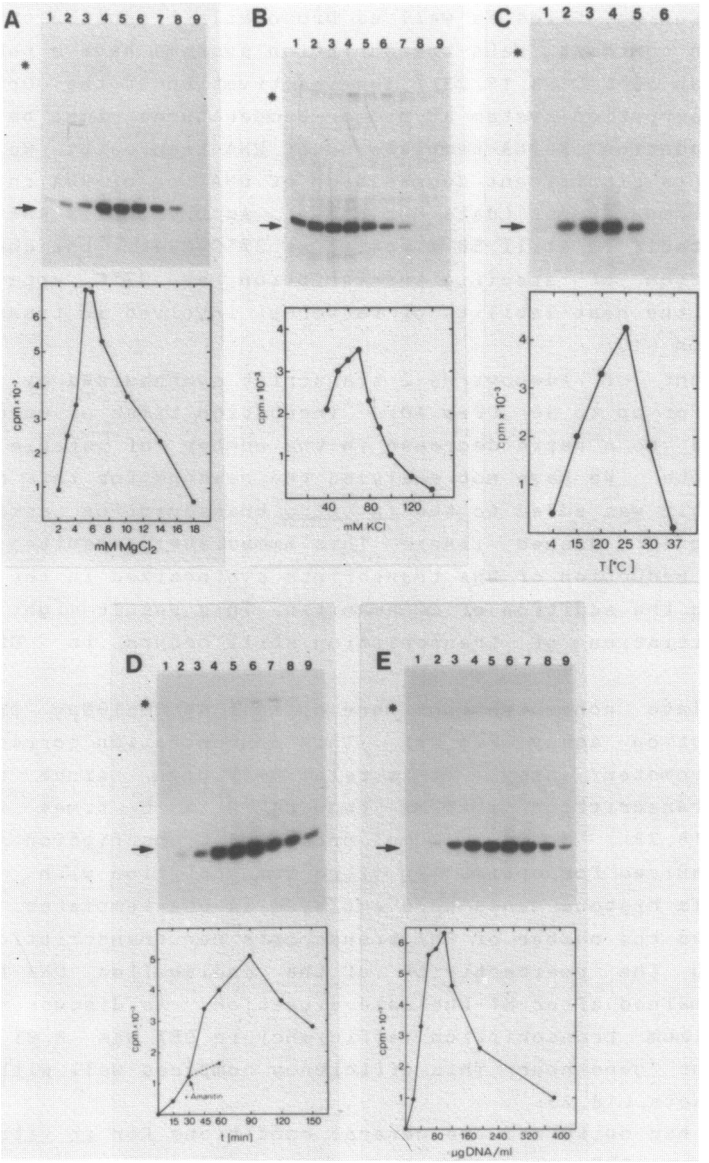

Fig. 4 Properties of the Drosophila in vitro transcription system. In vitro transcription from Adenovirus -2 major late promoter was assayed by S1-nuclease protection experiments (16) with a 5'-endlabelled XhoI-HindIII fragment of pAdomal (specific activity 3 x 10^4 cpm/pmol 5 '-end). Upper panel: PAGE of S1-nuclease protected DNA-fragments. The asterix indicates the original size of the endlabelled DNA, the arrow the 197 nucleotides long protected DNA. Exposure times of dried gels were 1hr. Lower

panel: Cerenkow-counts of the protected DNA-fragments of the corresponding upper panel. In vitro transcription assays were according to the protocol in Materials and Methods except for the parameter indicated. A. Lanes 1-9 correspond to transcription assays at 2, 3, 4, 5, 6, 7, 10, 14, and 18 mM $MgCl_2$. B. Lanes 1-9 correspond to transcription assays at 40, 50, 60, 70, 80, 90, 100, 120, and 140 mM KCl. C. Lanes 1-6 correspond to transcription assays at 4°C, 15°C, 20°C, 30°C, 37°C, D. Lanes 1-8 correspond to transcription assays for 0', 30', 45', 60', 90', 120', and 150'. Lane 9 corresponds to a transcription assay for 60', if 8 µg/ml α-amanitin was added at 30'. E. Lanes 1-9 correspond to transcription assays at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0 and 10 µg pAdomal DNA/assay.

H3/H4-DNA as well as 3'- truncated H2a/H2b-DNA as described in Materials and Methods (Fig. 1). Specific transcription of histone genes was measured by S1 nuclease protection experiments. The transcription assays had to take in account the presence of endogenous histone mRNA in DEX. Therefore, we have carried out one transcription assay with pAT153 vector alone and have analyzed in the reaction mixture the protection of the respective endlabelled DNA fragments against S1-nuclease digestion by endogenous histone mRNA (Fig. 5 A, lane 5). The construction of histone gene recombinants employed the addition of linker sequences as described in Materials and Methods. Thus, endlabelled DNA fragments, which were used for S1-nuclease protection experiments, had 5'-ends, which did not exactly match with in vivo histone mRNA. Extract-endogenous histone mRNA, therefore, could not efficiently protect the endlabelled DNA-fragments against S1-nuclease digestion. A comparison of lanes 1 and 2 of Fig. 5B illustrates this point. The XhoI-BglI-fragment of pAWB contains a 3'-deleted H2a gene, which exactly matches with the corresponding 5'-part of the H2a transcript. 2 µg total embryo RNA were sufficient to map the transcription start site from the H2a promoter. In contrast, 70 ug total embryo RNA were necessary to map the transcription start sites from the H2b, H3 and H4 promoters (lanes 2, 4, Fig. 5B) with endlabelled fragments of the pATH2a/b and pATH3/4 recombinants. These results showed that extract endogenous histone mRNA would not interfere in the S1-nuclease protection experiments with in vitro histone gene transcripts. The comparison of lanes 2 to 4 of Fig. 5A with lane 5, therefore, shows that DEX efficiently and accurately transcribed from histone genes H3, H4 and H2a. The data of Fig. 5A and B also indicate that transcription start sites from the histone gene promoters are very similar in vitro and in vivo. On the other

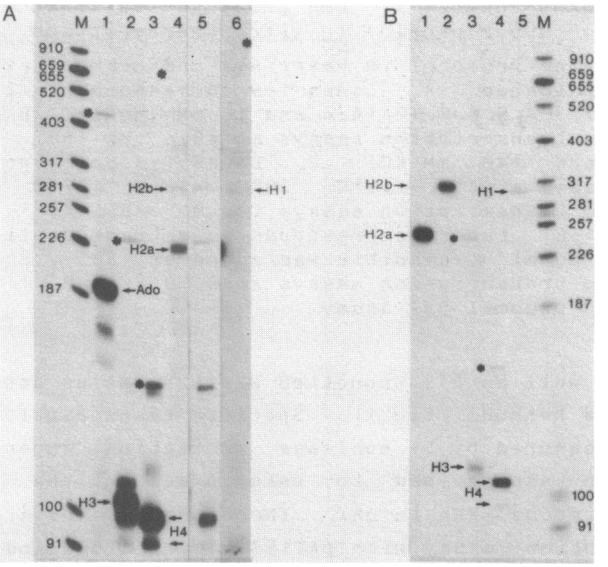

Fig. 5 In vitro transcription of Drosophila histone genes. In vitro transcription assays were as described in Materials and Methods. Transcripts were analyzed by S1-nuclease protection experiments (16). Endlabelled restriction fragments were prepared from the recombinants shown in Figure 1. Asterices indicate the size of the 5'-endlabelled DNA-fragments, arrows the expected fragment size protected by the corresponding gene transcript. Lanes M are DNA size markers. A. Templates and 5'-endlabelled fragments were : lane 1-2.5 µg pAdomal/assay and the XhoI-HindIII fragment (specific activity 6 x 10^6 cpm pmol 5 'end), lane 2 - 1µg pAT H3/4/assay and the HindIII-AvaI fragment (specific activity 6 x 10^6 cpm/pmol 5'-end), lane 3- 1 µg pATH3/4/assay and the AvaI-HindIII fragment (specific activity 6 x 10^6 cpm/pmol 5'-end), lane 4- 1 µg pATH2a/b/assay and the HindIII fragment (specific activity 3 x 10^6 cpm/pmol 5'-end), lane 5 - 2.5µg pAT 153/assay and the HindIII-AvaI-, the AvaI-HindIII- together with the HindIII fragment, lane 6 - 2 µg pAT H1/assay and the HpaI/HindIII - fragment (specific activity 4 x 10^6 cpm/pmol 5'end). Exposure time of the gel was 2hr. B. Mapping in vivo start sites of histone mRNA of 12hr old Drosophila embryos by S1-nuclease digestion. Lane 1-2µg RNA was mapped with 5'-endlabelled XhoI-BglI-fragment of pWAB, lane 2 - 70 µg RNA with the HindIII-fragment of pATH2a/b lane 3 - 70 µg RNA with the HindIII-AvaI-fragment of pAT H3/4, lane 4 - 70 µg RNA with the AvaI-HindIII fragment of pAT H3/4, lane 5- 135µg RNA with the HpaI-HindIII fragment of pATH1. Asterices indicate the sizes of endlabelled fragments (specific activities 1.1-1.5 x 10^6 cpm/pmol 5'-end) before, arrows after S1-nuclease digestion.

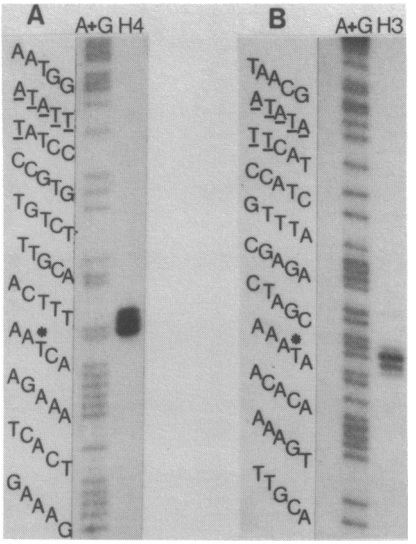

Fig. 6 Mapping in vitro transcription start sites from histone H3 and H4 promoters. In vitro transcription with pATH3/4 as template and S1-nuclease protection with H3- and H4-transcripts was carried out as in Fig. 5A. A. 1000cpm of S1-nuclease resistant material was electrophoresed together with pAT H3/4, which had been 5'-end-labelled at the HindIII site and had been cleaved at the purine residues according to (17). The H4 transcription start site is indicated by an asterix above the sequence of the left. The TATA-box element was underlined . B. 1000 cpm of S1-nuclease resistant material corresponding to the H3-transcript was electrophoresed together with pATH3/4, which had been 5 '-endlabelled at the AvaI-site and had been cleaved at the purine residues according to (17). The H3 transcription start site is indicated by an asterix above the sequence on the left. The TATA-box element was underlined.

hand, transcription from histone genes H1 and H2b could not be detected (lanes 4 and 6 of Fig.5A). The results were very similar to the mock transcription with pAT153 (lane 5 of Fig. 5A).
We have analyzed in vitro transcription from these genes in more detail. Lanes 3 an 4 of Fig.5A suggested that transcriptions from the H3 and H4 promoters were initiated at a major site and at a minor site, which are 5 bases upstream (H3) or 10 bases downstream (H4) of the major transcription start site. We also detected the minor transcription site for in vivo H4mRNA synthesis Fig.5B, lane 4), but not a second start site for in vivo H3 mRNA. The major transcription start sites were identical with the in vivo transcription start sites of H3 and H4 mRNAs (Fig.6).

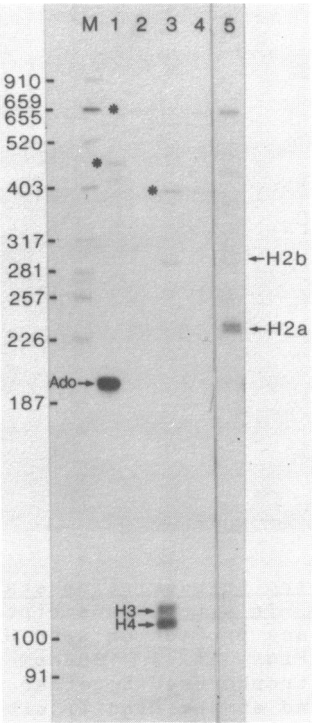

Fig.7 in vitro transcription from Drosophila histone gene promoters in HeLa WCE. Plasmids were transcribed in a HeLa WCE as described in Materials and Methods. Templates were in lane 1 pAdomal, lane 2 and 5 pATH2a/b, lane 3 pATH3/4, lane 4 pAT153. M: DNA size markers. S1-nuclease protection experiments were carried out in lane 1 with 5'-endlabelled XhoI-HindIII-pAdomal fragment (spec. activity 2 x 10^6cpm/pmol 5'-end), lanes 2 and 5 with 5'-endlabelled Hind III-pATH2a/b fragment (spec. activity 4 x 10^5cpm/pmol5'-end), lane 3 with 5'-endlabelled HindIII-pATH3/4 fragment (spec.activity 2 x 10^6 cpm/pmol 5'-end), lane 4 with an equimolar mixture of all fragments used in lanes 1-3. Exposure time of gel was 5hr. Lane 5 was identical to lane 2, but was exposed for 48hr. Asterices indicate sizes of 5'-endlabelled DNA-fragments, arrows sizes of accurate transcripts.

The difference of 1,5 nucleotides between the S1-nuclease protected DNA-fragments and the H3/H4 - start sites determined by chemical sequencing, is because the chemical DNA-sequencing method removes the 3'-base and 3'-phosphate of the labelled DNA-fragment (27). This inadvertantly generates DNA-fragments, which differ by 1,5 nucleotides from S1-nuclease generated fragments. Fig.6 also shows that the major H3 and H4-transcripts

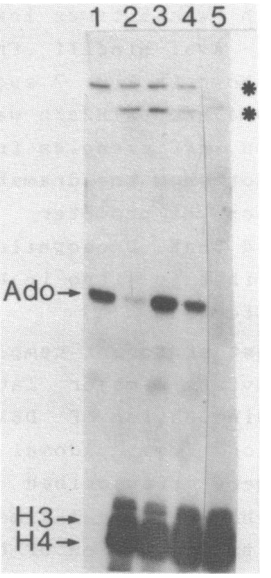

Fig. 8 Competition of in vitro transcription from pAdomal with pATH3/4. pAdomal and pATH3/4 were transcribed in DEX as described in Materials and Methods. Transcripts were analyzed by S1-nuclease protection experiments as in Figs. 2, 5, 7. Spec. activity of the 5'-endlabelled XhoI-HindIII-pAdomal fragment was 2.4 x 10^6 cpm/pmol 5'-end and of the HindIII-pATH3/4 fragment 1.7 x 10^6 cpm/pmol 5'-end. Fragment sizes are indicated by an asterix. Exposure time of the gel was 2hr. Lane 1: transcription from 1.5 µg pAdomal, lane 2: 10' preincubation of DEX at 25°C with 1 µg pATH3/4 before addition of 1.5 µg pAdomal as second template, lane 3: 10' preincubation of DEX at 25°C with 1.5 µg pAdomal before addition of pATH3/4 as second template, lane 4: transcription of 1 µg pATH3/4 together with pAdomal, lane 5: transcription from 1 µg pATH3/4.

varied by +/- 2 (H3) or by +1 to+2 (H4) nucleotides. Our experiments could not decide whether these microheterogeneities were an S1-nuclease digestion artefact (nibbling) or whether they were in fact due to inaccurate initiation of transcription by RNA polymerase B.

The results of Fig. 5 were compared with the transcription from Drosophila histone promoters in a HeLa WCE (18) (Fig. 7). Transcription from the pAdomal promoter was generally in WCE more efficient than from the Drosophila histone promoters. The pATH1 template was again inactive (data not shown). H2a/H2b transcripts were only detectable after prolonged autoradiography (Fig. 7, lane 5). However, considering the specific activity of the pATH2a/b-

HindIII fragment, which was 5 times lower than the specific activity of the pATH3/4 - AvaI-HindIII fragment, the S1-nuclease protection experiments shown in Fig. 7 suggest that transcription efficiencies from pATH3/4 and pATH2a/b were actually quite similar. Thus, transcription efficiencies from Drosophila histone promoters in WCE did not show the dramatic differences seen in DEX except for the histone H1 promoter. The comparison of DEX with HeLa WCE indicated that Drosophila H3 and H4 genes were more efficiently transcribed in vitro in DEX , i.e. in the homologous transcription system.

Since pATH3/4 was the most efficient template in the DEX system, transcription from Adenovirus-2 major late promoter was competed with pATH3/4. Preincubation of DEX with pATH3/4 inhibited completely transcription from pAdomal (Fig. 8). Even, if pATH3/4- and pAdomal were transcribed without preincubation, transcription from of the Adenoviral-2 major late promoter was 60 % reduced. However, preincubation of DEX with pAdomal did not inhibit transcription from pATH3/4. This indicated that the Drosophila factors, which were necessary for initiation of transcription from the Adenovirus-2 major late promoter, preferentially bound to the Drosophila H3 and (or) H4 promoter sequences. Therefore, we separated the H3/H4 transcription start sites by subcloning (see Materials and Methods) such that the H3-subclone contained most of the spacer region (104 bp upstream of the H3-TATA-box). The new H4 subclone had left only 22 bp upstream of the H4-TATA-box, as indicated in Fig. 1. The H3 and H4 subclones (pATΔH3 and pATΔH4) were transcribed with an efficiency equal to pATH3/H4, when identical promoter concentrations were employed (compare Figs. 5, 8 and 9). Separation of the H3- and H4-transcription start sites did not apparently alter in vitro transcription efficiencies at comparable promoter concentrations (0,37pmol of each promoter/assay). Surprisingly, however, pATΔH3 and pATΔH4 competed differently with transcription from pAdomal (Fig. 9) Although preincubation of DEX with pATΔH3 and pATΔH4 inhibited transcription from pAdomal as before (Fig. 9, lane 2), now, preincubation of the DEX with pAdomal also inhibited transcription from the histone H3 and H4 promoters (Fig. 9, lane 1). This inhibition was more pronounced for transcription from pATΔH3 (80 % reduction) than for transcription from pATΔH4 (30 % reduction), albeit the fact that pATΔH3, but not pATΔH4 possessed almost the entire intergenic spacer sequence (104 bp out off 126 bp, Fig. 1).

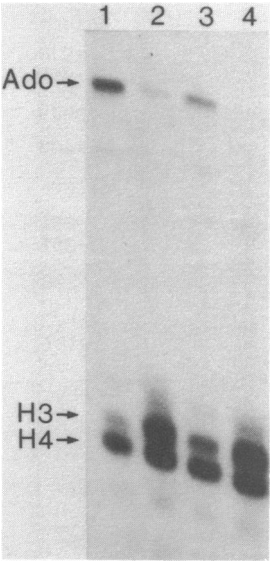

Fig. 9 Competition of in vitro transcription from pAdomal with pAT Δ H3 and pAT Δ H4. Plasmids were transcribed in DEX as described in Materials and Methods. Transcripts were analyzed by S1-nuclease protection experiments as in Fig. 8. Exposure time of the gel was 1.5hr. Lane 1: 10' preincubation of DEX at 25°C with 1.5 µg pAdomal before addition of 0,6 µg pAT ΔH3 and 1.3 µg pAT Δ H4. (This is equivalent to 1 µg pATH3/4. It corresponds to 0,37 pmol H3- and 0,37 pmol H4-promoter/assay), lane 2: 10'-preincubation of DEX at 25°C with 0.6 µg pATΔH3 and 1.3 µg pAT ΔH4 before addition of 1.5 µg pAdomal, lane 3: transcription from 0.6 µg pATΔ H3 and 1.3 µg pAT ΔH4 together with 1.5 µg pAdomal, lane 4: transcription from 0.6 µg pAT Δ H3 and 1.3 µg pAT Δ H4.

Finally, capping of H3/H4-transcripts was analyzed. In vitro transcripts were chromatographed on a dihydroxyboronyl-sepharose column, which retains capped RNA-molecules (20,21). The flow through of this column was hybridized with endlabelled AvaI-Hind III - pATH3/4-fragment in order to quantitate H3/H4 in vitro-transcripts by an S1-nuclease protection experiment and with endlabelled XhoI-BglI-pAWB fragment the H2a in vivo transcript, which is present in DEX as described above. The salt eluate of the dihydroxyboronyl-sepharose column was digested with Tobacco acid pyrophosphatase, which removes cap-structures of mRNA by hydrolyzing the pyrophosphate ester bond (22). The Tobacco acid pyrophosphatase treated material was rechromatographed on a second dihydroxyboronyl-sepharose column. Transcripts in the flow through and in the salt eluted material of the second column were

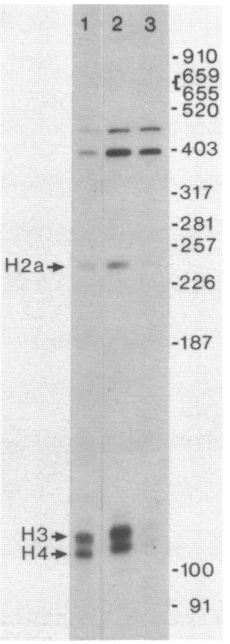

Fig. 10 Analysis of capping of in vitro H3/H4-transcripts. In vitro transcription from pATH3/4 was as described in Materials and Methods. In vitro transcripts were chromatographed on a dihydroxyboronyl-sepharose column (21). Salt eluted material was treated with Tobacco acid pyrophosphatase (22) and was rechromatographed. Flow throughs and salt eluates were then hybridized with 5'-endlabelled XhoI-BglI-pWAB-fragment (2 x 10^6 cpm/pmol 5'-end) and HindIII pATH3/4 fragment (5 x 10^5 cpm/pmol 5'-end) and S1-nuclease digested (16). S1-nuclease protected DNA-fragments of the first flow through (lane 1), of the second flow through after pyrophosphathase treatment (lane 2), of the second salt eluate after pyrophosphatase treatment (lane 3). Arrows indicate accurate transcript sizes. DNA size markers were as in the previous Figures.

again quantitated by S1-nuclease protection experiments. The data in Fig. 10 show that the first dihydroxyboronyl-sepharose-column retained most of in vitro (H3/H4) and in vivo (H2a) histone RNA (70 % of the applied material). After pyrophosphatase treatment, decapped transcripts now adsorbed no longer to the second dihydroxyboronyl-sepharose-column and quantitatively flowed through the column (Fig. 10, lanes 2 and 3). In vivo H2a transcripts yielded similar results as in vitro H3- and H4-transcripts in this analysis. This suggests that DEX produced capped transcripts. The exact number and structure of the capped transcripts remains to be determined.

DISCUSSION

A Drosophila Polymerase B transcription system has recently been developped by extracting nuclei of Drosophila tissue culture cells (12). This procedure requires large quantities of tissue culture cells, which is time consuming and quite expensive. Drosophila embryos, on the other hand, are an easily obtainable and cheap starting material. Both transcription systems apparently have the same overall properties in terms of salt and temperature requirements (12) (Fig. 4), although a detailed characterization of the tissue culture system has not yet been published. Three differences, however, are noteworthy. DEX works well with circular DNA-templates. But in contrast to the tissue culture extract, we have not been able yet to prepare an exonuclease-free extract, which could utilize linear DNA-templates to study the synthesis of run-off transcripts. Therefore, specific transcription had to be principally assayed by S1-nuclease protection experiments (16). Secondly, DEX requires relatively high concentrations of DNA-template, i.e. 0,75-1,0 pmol promoter/assay. The efficiencies of transcription from the various promoters can only be estimated, since a direct analysis of run-off transcripts was not possible. The most conservative estimate is based on the assumption that the hybridization efficiency between RNA-transcript and endlabelled DNA-probe was 100 % in the S1-nuclease protection experiments. Accordingly, 0,01 pAdomal, respectively, 0,04 H3/H4 transcripts/gene/hour were synthesized *in vitro* in DEX. This efficiency of *in vitro* transcription compares well with HeLa-transcription systems (18, 23). It is 10 times higher in the Drosophila tissue culture extract (12). The requirement of transcription in DEX for high DNA-concentrations might be due to unspecific binding of proteins to DNA-template, which covers DNA-binding sites for transcription initiation factors (28). This effect of non-specific DNA binding proteins has been compensated for in other transcription systems by adding non-template DNA to the transcription assay (23, 29, 30). Thus, the amount of transcript synthesized per gene and hour was increased. Similarly, we have tried to increase the efficiency of specific transcription in DEX by substituting template-DNA with varying amounts of non-template (vector) DNA. These attempts have failed so far (data not shown). Instead of the amount of transcript/gene/hour we have computed the amount of specific transcript per assay or 15ul DEX, respectively. This computation was again based on the assumption that the hybridization efficiency of RNA-transcript with

endlabelled DNA-fragment was 100 % in the S1-nuclease protection experiments. We obtained 1 x 10^{-2} pmol pAdomal- and 2 x 10^{-2} pmol H3/H4-transcript/assay. These numbers are similar to the 3 x 10^{-2} pmol H3/H4-transcript/assay synthesized in the Drosophila tissue culture extract (12). 1,5 to 4 x 10^{-3} pmol Adenovirus transcript/assay have been synthesized with HeLa-transcription systems (18, 23). This suggets that Drosophila transcription systems are more efficient than HeLa-systems. Thirdly, transcripts were synthesized in vitro from the H2a-promoter in DEX, but not in the Drosophila tissue culture extract (12). Since identical histone-templates, which originate from the cDM 500 clone (26), have been used, DEX apparently contains essential factor(s) for transcription from the H2a promoter which the tissue culture extract lacks. Clustering of histone genes in the genome suggested a coordinate expression of the histone genes at the level of transcription (31). Transcriptions from H1, H2a, H2b, H3 and H4 promoters apparently required in vitro specific factors, which differently regulate the initiation of transcription from either gene. The efficiency of in vitro transcription from the histone promoters decreased in DEX in the order H4>> H3> H2a>> H2b, H1 (Fig. 6). This may indicate that factor(s) are absent in DEX for efficient transcription from H2b and H1 promoters and are not sufficient for efficient transcription from H2a.

Parker and Topol (12) have characterized a "transcription factor B" which binds to the "TATA"-box regions of histone genes H3 and H4. This factor stimulates in vitro transcription from the H3/H4 promoters. It also stimulates transcription from the 5C actin gene promoter (12). Therefore, this factor might be a more general transcription initiation factor. It is apparently necessary, but not sufficient for initiation of transcription from the H3/H4 promoters. The competition experiments between pAdomal- and pATΔH3/pATΔH4-templates showed that preincubation of DEX with pAdomal DNA inhibited transcription from the H3-promoter more strongly than from the H4-promoter, albeit most upstream sequences had been deleted . The H4-promoter sequence in pATΔH4 started at -22 base pairs upstream of the "TATA"-box. Thus, it just includes the 65 bp region of the H4-promoter, which transcription factor B protected against DNAseI digestion (12). Since the H3-promoter similarly interacts with transcription factor B, it should not be responsible for the result that transcriptions from pATΔH3 and from pATΔH4 were competed by pAdomal differently. Therefore, additional transcription initiation factors may be

involved, which could interact either at or, possibly, downstream of the H4-transcription start site.

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