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Chromatin structure and DNase I hypersensitivity in the transcriptionally active and inactive porcine tumor necrosis factor gene locus

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ABSTRACT

We have analyzed the chromatin structure of the porcine tumor necrosis factor gene locus (TNF-α and TNF-β). Nuclei from porcine peripheral blood mononuclear cells were digested with different nucleases. As assessed with micrococcal nuclease, the two TNF genes displayed slightly faster digestion kinetics than bulk DNA. Studies with DNaseI revealed distinct DNaseI hypersensitive sites (DH-sites) within the porcine TNF locus. Four DH-sites could be observed in the promoter and mRNA leader regions of the TNF-β gene. Two DH-sites could be observed for the TNF-α gene, one located in the promoter region close to the TATA-box and the other site in intron 3. This pattern of DH-sites was present independently of the activation state of the cells. Interestingly in a porcine macrophage-like cell line, we found that the TNF-α promoter DH-site disappeared and another DH-site appeared in the region of intron 1. Additionally, the DH-site of intron 3 could be enhanced by PMA-stimulation in these cells. TNF-β sites were not detected in this cell line. However, DH-sites were totally absent in fibroblasts (freshly isolated from testicles) and in porcine kidney cells (PK15 cell line) both of which do not transcribe the TNF genes. Therefore, the pattern of DH-sites corresponds to the transcriptional activity of analyzed cells.

INTRODUCTION

Tumor necrosis factors alpha and beta, also known as cachectin and lymphotoxin, respectively, are two structurally and functionally related cytokines with overlapping biological activities (for review see 1–4). Both cytokines play an important role in inflammation and early events of the immune response. TNF-α is mainly a product of stimulated monocytes and macrophages, whereas TNF-β is mainly produced in lymphocytes (2,5,6). Despite a similarity on the amino-acid level of only 30% (7), TNF-α and TNF-β bind to the same cell surface receptors. Up to date the sequences of human, mouse, rabbit and swine TNF genes are known (8–13). The two genes are arranged in a tandem orientation in all species with TNF-β 5′ to TNF-α, separated by about 1 kb of intergenic DNA. Mapping of the TNF genes showed that they are closely linked to the MHC complex (14).

It has been shown that the TNF genes are regulated, at least in part, at the level of transcription and that this transcription is cell-type specific (15–18). Additionally, these genes are also regulated at posttranscriptional and (post)translational levels (19,20). The mechanisms and the chromatin structure governing this regulation is poorly understood. Positive regulation by NFkB has been implicated to play a role in the transcriptional regulation of TNF-α (21,22). Negative regulation of the TNF-β gene has been attributed to a sequence of the proximal promoter (23).

An indirect way to analyze the transcriptional regulation of the TNF genes is to analyze their basic structure, i.e. the chromatin structure, and relate this to the immunological function and transcriptional state of the cells. In this paper we report about the overall chromatin structure and the DH-site pattern that can be observed in nuclei isolated from porcine peripheral blood mononuclear cells, fibroblasts, the porcine kidney cell line PK15, and from a porcine macrophage-like cell line. The obtained results are compared and discussed in the context of transcriptional activity.

MATERIALS AND METHODS

Isolation of porcine mononuclear cells and pig testicle cells

Peripheral blood mononuclear cells (PBMC) were obtained from freshly drawn blood of healthy pigs. The blood was collected in an autoclaved silanised glass-flask containing EDTA as the anticoagulant (7.5 mM final concentration). Blood was transferred to conical 50 ml polypropylene tubes (Nunc, Denmark) and allowed to equilibrate with room-temperature. After centrifugation (500×g, 10 min at room-temperature, no brake) in a Varifuge RF (Heraeus, Switzerland) buffy-coats were pooled and washed three times with citrate-buffer (30 mM citrate, 4.8 mM glucose, 3 mM KCl, 0.9% NaCl, pH 6.5). Separation of PBMC from other blood cells was achieved by isopycnic centrifugation on Ficoll-Hypaque™ (d= 1.077, Seromed, Biochrom KG, Germany). The PBMC-phase was washed three

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times with ice-cold phosphate-buffered saline (PBS: 10 mM Na-phosphate, 0.9% NaCl, pH 7.4) supplemented with 0.3 mM EDTA. Cells were counted, adjusted to 5×10^7/ml in PBS and kept on ice for further use.

The testicle cell culture was obtained from testicles of piglets. Briefly, after surgery, testicles were put on ice under sterile conditions and were cut into pieces. The pieces were stirred for 30 min at 37°C in a trypsin/EDTA solution (0.05%/0.02% [w/v], Seromed), filtered through a sterile cheesecloth and cells were seeded on polystyrene flasks (Falcon) in minimal essential medium (MEM, Flow Laboratories, Scotland) supplemented with 2 mM glutamine (BioMérieux, France), 100 U/ml penicillin (Seromed), 100 μg/ml streptomycin (Seromed) and 20% fetal bovine serum (FBS, SEBAK, Germany). The culture was incubated in a humidified incubator with 5% CO_2 at 37°C. Cells were passaged at confluency. After the third passage cells were no longer viable.

Cell lines

A porcine kidney cell line (PK15, ATCC no.: CCL 33) was cultured in MEM supplemented with 7% FBS. Cells were harvested from culture flasks by trypsinization.

The porcine macrophage-like cell line was a gift from Mr. Tschudin (Institute for Viral Disease and Immunoprophylaxis, Basel, Switzerland). Culture conditions were the same as for PK15. These cells were characterized by their ability to be infected with African Swine Fever virus. In addition the cells were shown to be positive for non-specific esterase a marker for monocytes and macrophages (24). However, this cell line was negative in a well established human phagocytosis assay (25) using sheep erythrocytes opsonized with rabbit-anti-sheep IgG, bovine-anti-sheep IgG or human erythrocytes opsonized with human-anti-D IgG.

Stimulation of cells with phorbol myristate acetate (PMA, Sigma) was overnight in cell culture medium (see above) containing 2.7×10^{-8} M PMA.

Isolation of nuclei

1 ml PBMC suspension (5×10^7 cells) was swollen in hypotonic buffer (10 mM Tris·HCl, pH 8.0/ 10 mM NaCl/ 5 mM $MgCl_2$) for 30 min on ice. After centrifugation (1400×g, 2 min) the cells were resuspended in hypotonic buffer and homogenized in a Dounce-homogenizer (loose pestle, 20 strokes) in the presence of 0.3% Nonidet P-40™ (Sigma). Nuclei were purified by centrifugation (1500×g, 10 min) through hypotonic buffer containing 8.5% sucrose (w/v) and resuspended in 1 ml digestion buffer (100 mM Tris-HCl, pH 8.0/ 50 mM NaCl/ 3 mM $MgCl_2$/ 1 mM $CaCl_2$). Nuclei were counted and adjusted to 2.5×10^7/ml and kept in 400 μl aliquots on ice until digestion.

Nuclei were isolated from fibroblast, PK15 and macrophage-like cells in the same way except that the homogenization of cells was carried out with 40 strokes.

Nuclease treatment

10^7 nuclei were digested with different amounts of either micrococcal nuclease (Pharmacia, Sweden) or bovine pancreatic DNaseI (Sigma) for 1, 2, 5 and 10 min at 37°C. Controls were an undigested, immediately lysed sample and a sample which was incubated at least for 10 min at 37°C without the addition of an exogenous nuclease. The digestion was stopped by transferring 100 μl of digested nuclei to 100 μl stop solution (200 mM Tris-HCl, pH 8.0/ 200 mM NaCl/ 20 mM EDTA/ 2% SDS/ 200 μg/ml proteinase K) and incubated for 2 hours at 37°C.

DNA purification and analysis

DNA was phenol/chloroform extracted and ethanol precipitated according to Sambrook et al. (26). The DNaseI treated samples were digested with different restriction endonucleases (Boehringer Mannheim, Germany) according to the recommendations of the supplier. DNA was separated on agarose gels and transferred to nitrocellulose (HybondC, Amersham) according to Sambrook et al. (26). DH-sites were detected by indirect end-labelling (27). Labelling of specific probes was done with a random oligonucleotide priming or with a nick-translation kit (Boehringer Mannheim, Germany). In order to minimize background, filters were boiled for 5 min in H_2O before hybridization. Hybridization was done according to Sambrook et al. (26) in plexiglas tubes over night in the presence of 50% formamide at 37°C. After hybridization, filters were washed four times for 15 min with 1× SSC/0.1% SDS at room-temperature, twice for 15 min with 0.1× SSC/0.1% SDS at room-temperature and twice for 15 min with 0.1× SSC/0.1% SDS at 65°C. Filters were then exposed to Hyperfilm™-MP (Amersham) at −70°C with an intensifying screen for up to 6 days.

RESULTS

Sensitivity of PBMC chromatin to micrococcal nuclease

It is well established that the nucleosomal pattern of actively transcribed genes is disrupted and that the kinetics of digestion is faster for active genes than for bulk DNA (28). In order to analyze the overall chromatin conformation of the porcine TNF locus, isolated nuclei from PBMC were subjected to digestion with micrococcal nuclease, transferred to nitrocellulose filters and probed subsequently with TNF-specific and genomic DNA. Analysis was performed by comparing the digestion pattern of the two TNF genes with that of genomic DNA (Fig. 1). The kinetics of digestion was slightly faster for the TNF-α and TNF-β genes than for bulk DNA. This effect could be seen best in the 2 min digest (Figure 1A, 1B, 1C, lane 4) where the TNF-DNA was digested to smaller fragments (A,B) than genomic DNA (C). Also, the dinucleosomal band (lanes 5 and 6) was more intense

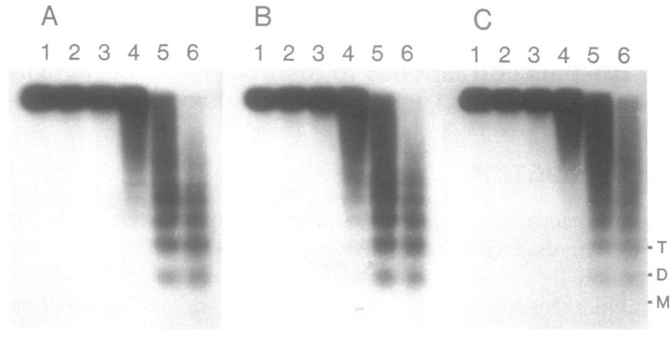

Figure 1. Sensitivity of porcine TNF genes to micrococcal nuclease. Isolated nuclei from porcine PBMC were digested with micrococcal nuclease. Purified DNA was separated on a 2% agarose gel, transferred to nitrocellulose and subsequently hybridized with different probes. *Panel A:* TNF-α. *Panel B:* TNF-β. *Panel C:* genomic DNA. Exposition was 2 days (A), 3 days (B) and 30 min (C) with an intensifying screen at −70°C. Lanes 1−6: control not incubated, incubated control, 1, 2, 5 and 10 min digestion with 11 U/ml micrococcal nuclease in the presence of 5 mM $CaCl_2$. M, D, T: position of nucleosome monomer, dimer and trimer bands, respectively. The absence of the monomer band is due to a filter artifact.

for TNF-α (A) and TNF-β (B) than for bulk DNA (C). However, the nucleosomal ladder displayed an identical repeat length of 194.6 ± 6.7 bp in all three autoradiographs, indicating that the higher sensitivity of the TNF genes to micrococcal nuclease was not caused by differences in repeat length between nucleosomes.

DNaseI hypersensitivity within the TNF locus of porcine PBMC

Overall DNaseI hypersensitivity. In order to search for DH-sites in the porcine TNF locus, isolated nuclei from PBMC were digested with limited amounts of DNaseI and the isolated DNA was digested with HindIII. This fragment covers the entire TNF locus (Fig. 2B). DH-sites were detected by means of Southern-transfer and indirect end labelling.

At this resolution at least one site located within the TNF-α promoter region (Fig. 2A, α) and one or more sites within the TNF-β promoter region could be observed (Fig. 2A, β). Band x in Fig. 2A represented a region of DNaseI-hypersensitivity that lay outside of our cloned TNF locus and was therefore not further analyzed.

Some of the DH-sites which could be observed were not very intense compared to other DH-sites reported in the literature (29−31) indicating that only a subpopulation of the cells possessed these sites.

DH-sites within the TNF-α gene. In order to obtain more precise information about the location of DH-sites in the TNF-α gene, nuclei of PBMC were digested with limited amounts of DNaseI and the purified DNA was digested with EcoRI prior to Southern-blotting. The EcoRI digestion of genomic DNA resulted in a 3.5 kb fragment containing 5′ and 3′ untranslated sequences in addition to the TNF-α coding region. Fig. 3A displays results obtained by indirect end-labelling of the 3′ end of the EcoRI restriction fragment. Mapping of the bands showed one site located in the promoter region close to the position of the TATA-box (Iα). This region extended over 120 bp. The other site (IIα) was precisely located within intron 3 and extends over 150 bp. Site IIα smeared after a certain time of DNaseI digestion towards the 3′ end of intron 3, but never into the region of exon 4. This fact indicates that the DH-site towards this end of intron 3 was somewhat more resistant to DNase I. Interestingly an Sp1 and an AP-1 binding motif are located close to the border of intron 3 to exon 4.

Both DH-sites were reproducibly detected in freshly isolated PBMC from 9 individual animals. The same two sites were also mapped from the opposite end of the EcoRI fragment and were localized at identical positions and with the same extensions (data not shown).

DH-sites within the TNF-β gene. Similarly, the DH-sites in the TNF-β gene were resolved at higher resolution. Purified DNA of DNaseI treated PBMC nuclei was digested with EcoRI, resulting in a 2.3 kb restriction fragment including 3′ and 5′ untranslated and coding regions of TNF-β. After Southern-transfer to nitrocellulose filters, these were hybridized with a probe binding to the 3′ end of the EcoRI fragment containing the TNF-β gene. This experiment revealed four distinct DH-sites (Fig. 3B) which could be mapped within the promoter region (Iβ and IIβ) and within the mRNA leader sequence of the TNF-β gene (IIIβ and IVβ). Sites Iβ and IIIβ were weak bands, IIβ and IVβ were observed as strong bands in all experiments. Whereas sites Iβ and IVβ spanned a region of about 100 bp, site IIβ was 150 bp and site IIIβ extended over only 50 bp. Mapping the sites from the other end of the clone could not be performed because this end contained a repetitive sequence (9).

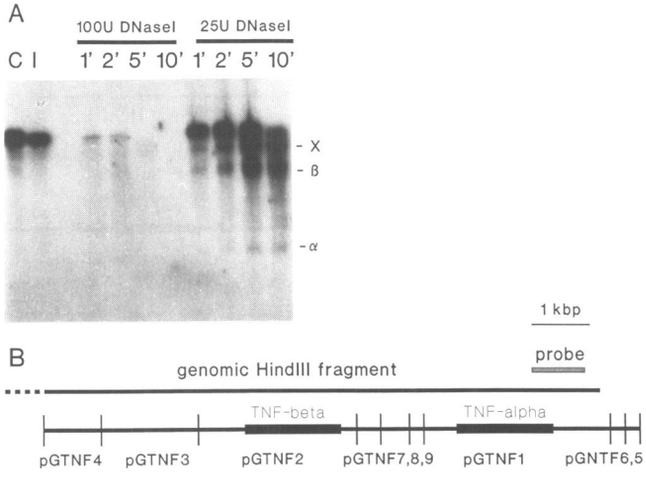

Figure 2. Overall DH-sites in the porcine TNF locus. Nuclei from PBMC were digested with DNaseI and the purified DNA was digested with HindIII. Fragments were separated on a 1% agarose gel and transferred to nitrocellulose filter. *Panel A:* Lanes C and I: unincubated (C) and incubated (I) controls, respectively. Digestion time and DNaseI concentration are indicated at the top. Bands indicating DH-sites are assigned x, α (TNF-α region) and β (TNF-β region). *Panel B:* Map of HindIII restriction fragment and probe used for indirect end-labelling. The map corresponds to the published sequence of the porcine TNF locus (9). pGTNF1-9 correspond to EcoRI fragments of the porcine TNF locus. Coding regions for both genes are indicated by a black bar. The dotted line indicates that the HindIII fragment extends outside to the cloned fragment (9).

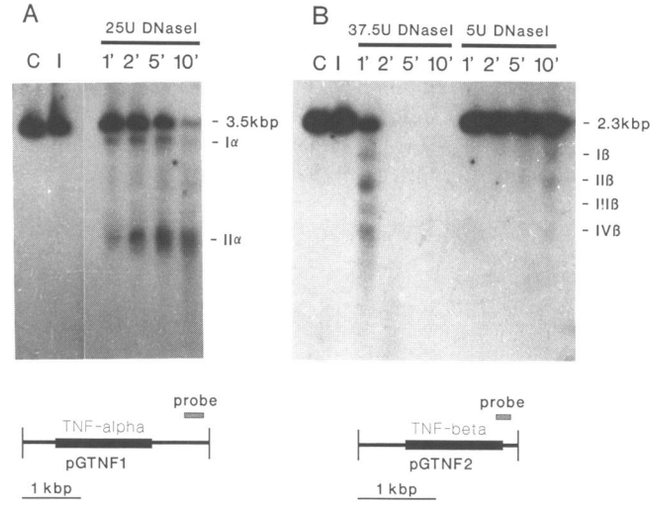

Figure 3. DH-sites within the TNF-α and TNF-β genes. Nuclei were digested with different amounts of DNaseI. The purified DNA was digested with EcoRI and separated on 1% agarose gels. After Southern-transfer filters were hybridized. Specific probes and analyzed fragment are indicated below the autoradiograph. *Panel A:* DH-sites within the TNF-α gene. Lanes C and I: not incubated and incubated controls, respectively. Digestion time and DNaseI concentration are indicated at the top. Size of restriction fragment and DH-sites are shown on the right. *Panel B:* DH-sites within the TNF-β gene. Lanes C and I are the same as in panel A. Digestion time and DNaseI concentration are indicated at the top. Size of restriction fragment and DH-sites are shown on the right.

The intensity of the DH-sites of TNF-β is reflecting varying accessibility to DNaseI, suggesting at least two different types of chromatin structure at the four sites.

DH-sites between the TNF genes. In addition to the DH-sites showed in Fig. 2 and designated x, α and β we analyzed the weak bands seen between sites α and β in Fig. 2. These are located between the two TNF genes. To study this region we digested the purified DNA of DNaseI treated PBMC nuclei with BamHI. This digestion resulted in a fragment covering the entire linker region between the two genes and the entire TNF-β gene. By means of indirect end-labelling of the 3′ end of this fragment, it was not possible to detect any other DH-sites besides the ones already observed for TNF-β (data not shown).

In summary, with porcine PBMC we found two distinct DH-sites within the TNF-α promoter (Iα) and intron region (IIα), four distinct DH-sites within the TNF-β promoter and mRNA leader sequence (Iβ–IVβ) and one region of DNaseI hypersensitivity about 1 kb 5′ outside of our sequenced porcine TNF clone (Fig. 2, band x).

Occurrence and induction of DH-sites in different cell types

To correlate the appearance of the DH-sites with function, we analyzed their inducibility and/or cell-specificity. For this purpose we chose five different approaches:

i) Comparison of unstimulated and LPS-stimulated PBMC.
ii) Comparison of separated monocytes and lymphocytes.
iii) Comparison of the DH-site pattern of an unstimulated and PMA-stimulated primary cell culture (fibroblasts) with that of PBMC.
iv) Comparison of the DH-site pattern of an unstimulated and PMA-stimulated porcine macrophage-like cell culture.
v) Comparison of the DH-site pattern of an unstimulated and PMA-stimulated established kidney cell line with that of PBMC and the macrophage-like cell line.

LPS stimulation of PBMC and comparison of monocytes and lymphocytes. Since LPS is a potent stimulus for TNF-α production in monocytes, we compared LPS-stimulated and unstimulated porcine PBMC for their pattern of DH-sites. Probing for the TNF-β and the TNF-α genes, it was not possible to observe any difference in the DH-site pattern between stimulated and unstimulated cells (data not shown). In contrast, we found increased levels of mRNA upon stimulation with LPS, suggesting

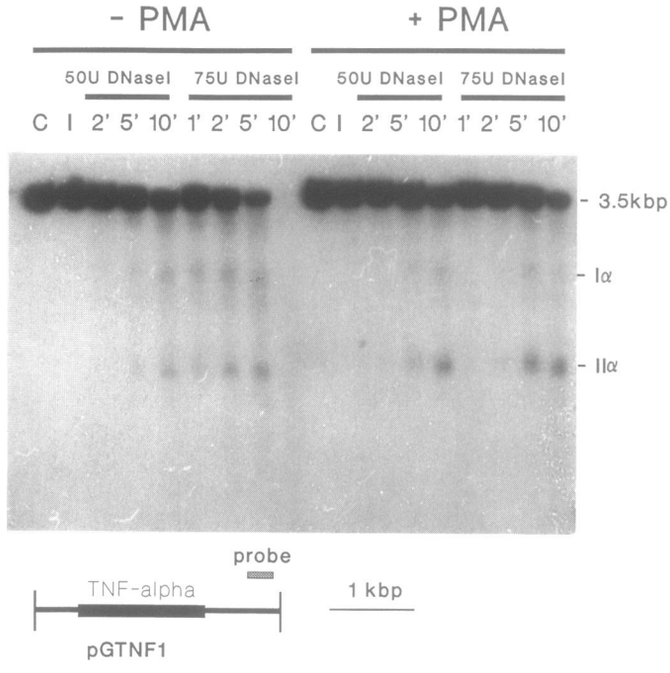

Figure 4. DH-sites within the TNF-α gene upon stimulation of swine macrophages-like cells with PMA. Cells were stimulated overnight with PMA. Nuclei from unstimulated and stimulated macrophage-like cells were digested with DNaseI. Purified DNA was digested with EcoRI, separated on a 1% agarose gel and transferred to a nitrocellulose filter. Specific probes and analyzed fragment are indicated below the autoradiograph. Controls (C and I) as in previous figures. Digestion time and DNaseI concentration are indicated at the top. Size of restriction fragment and DH-sites are shown on the right.

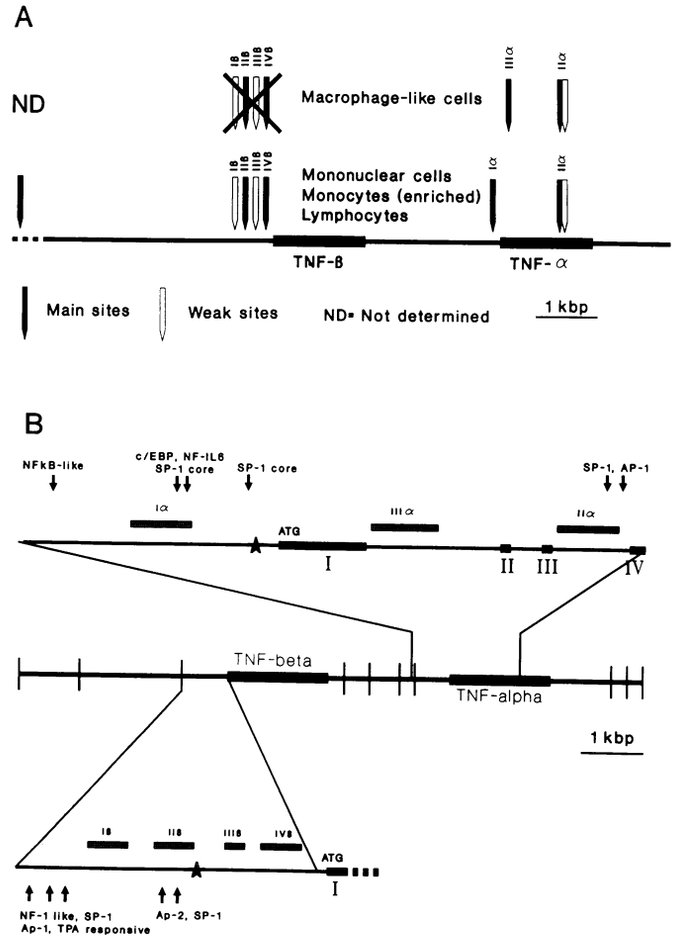

Figure 5. Comparison of observed DH-sites within the porcine TNF locus in different cells. *Panel A:* Arrows indicate the position of DH-sites according to the published sequence (9). ND indicates that this site was not analyzed in macrophage-like cells. Coding regions are indicated by a solid bar. *Panel B:* Top: Detailed view of the promoter and coding region of the TNF-α gene. Bottom: Detailed view of the promoter region of the TNF-β gene. Arrows pointing up and down indicate transcription factor binding sequences. Black bars indicate coding regions of the two genes. Roman numbers for TNF-α and TNF-β indicate the exons. ATG: translation start site. Asterisk: putative transcription start site. Hatched bars indicate the position of the observed DH-sites.

increased transcription or alternatively, altered mRNA-stability (data not shown).

In a second approach we analyzed the DH-site patterns of separated monocytes and lymphocytes. Freshly isolated PBMC were separated by adherence to polystyrene flasks. By this method we achieved a 90% enrichment of monocytes in the adherent cell fraction, whereas most of the non-adherent cells were lymphocytes. The DH-site pattern characteristic of PBMC was present for both the TNF-α and TNF-β genes and no obvious difference could be observed between the two cell populations (data not shown).

DH-sites within the TNF genes in fibroblast cells and in kidney cells. In another series of experiments we analyzed the DH-site pattern of primary testicle cell cultures (a primary cell culture containing mainly fibroblasts) and of the porcine kidney cell line PK15 (an established cell line). With fibroblasts we had a cell type other than PBMC in which the TNF genes should be silent and which was easy to cultivate (32).

Both cell types were also stimulated with PMA in order to activate the TNF genes. DH-sites could not be observed in any experiment neither in unstimulated nor in PMA-stimulated PK15 or testicle cell-cultures (data not shown).

Induction of DH-sites in porcine macrophage-like cells. Using porcine macrophage-like cells we investigated if these cells could be stimulated with PMA for TNF-α and/or TNF-β production and if such a stimulation had an influence on the chromatin structure of the TNF genes. For TNF-α the observed pattern of DH-sites was quite different from that of PBMC (Fig. 4). Site Iα was no longer present but another site could be mapped in intron 1 (IIIα). Site IIα was still present but seemed to be consistently enhanced when the cells were stimulated with PMA. In contrast to PBMC, the TNF-β gene displayed no DH-sites (data not shown), which is in good agreement with the absence of TNF-β transcription in macrophages (33). The exact structure and functional implications of the changed DH-site pattern and the enhanced DH-sites is not clear at the moment and will be further investigated.

DISCUSSION

Our results point to a complex structure/function relationship suggested by the different DH-site patterns in the different cells analyzed. Fibroblasts from testicles and kidney cells in which the TNF genes are silent did not display any DH-site pattern. In a macrophage-like cell line where only the TNF-α gene should be active, we could detect only DH-sites for this gene. However, in PBMC where both genes are active we could observe a DH-site pattern for both TNF genes. A schematic overview of DH-sites within the porcine TNF locus in the various cell types is given in Figure 5A.

The following conclusions can be drawn from our results. *First*, the overall sensitivity to micrococcal nuclease was higher for TNF-α and TNF-β than for genomic DNA, indicating a more open chromatin structure of the TNF genes. *Second*, no DH-sites were observed in freshly isolated fibroblasts and in an established porcine kidney cell line. Since these cells do not transcribe TNF (32) we suggest that the absence of DH-sites reflects the structural aspect of the inactive state of the TNF genes. *Third*, freshly isolated PBMC and macrophage-like cells displayed a pattern of DH-sites which can be correlated to the transcriptional activity of the TNF genes in these cells. *Fourth*, although TNF-α and TNF-β are related genes, their DH-site pattern was different. Whereas the TNF-α gene displayed two sites, one in the promoter and one in intron 3, the TNF-β gene displayed 4 sites, all of which were located in the promoter region and mRNA leader region. These DH-sites probably reflect the different regulation of the two genes, as was already postulated after the sequencing of the porcine TNF genes (9). *Finally*, an effect of LPS- or PMA-stimulation was only observed in the macrophage-like cells where the DH-site in intron 3 of the TNF-α gene seemed to be enhanced.

In our system we have analyzed freshly isolated PBMC *in toto* or separated in monocyte- and lymphocyte-enriched fractions. In these cells which are known to transcribe the TNF genes (34,35) we have observed a distinct DH-site pattern. However, in fibroblasts and kidney cells which do neither express TNF-α nor TNF-β the DH-site pattern is absent. In macrophage-like cells where the TNF-α gene should be active and the TNF-β gene should be silent we could only observe DH-sites for the TNF-α gene.

Although the resolution of our approach is only ±50 basepairs it is interesting to note that sites Iα, IIα, Iβ and IIβ map at positions in the sequence, where we have found possible transcription factor binding sites (9). Fig. 5B gives a detailed overview of the DH-sites in correlation to the sequence and possible transcription factor binding sites.

There is accumulating evidence that introns play a role in the regulation of transcriptional events (36). Comparison of intron 3 of TNF-α between human, rabbit and mouse compared to pig demonstrates that it is the most conserved one, i.e. 85%, 78% and 72%, respectively. Within one species all the other introns are on average about 10% less conserved (9). This fact, together with the mapped DH-site IIα suggests that intron 3 plays a role in the regulation of TNF-α transcription. We are currently investigating this hypothesis by expressing cDNA- and genomic DNA-clones in eukaryotic cells.

Another interesting point is the shift of site IIIα into the intron 1 in macrophage-like cells. It might indicate that transcription factors are absent in the promoter region of TNF-α or that the gene is differently regulated in this differentiated state of the cell.

TNF-α is regulated, at least in part, at the level of transcription (6,17,21). In resting monocytes no mRNA was detected in run-on experiments (15). However, this state is readily modulated to a high transcription rate within 20 minutes by PMA-stimulation. In addition, it seems that TNF-α expression is not restricted to any special type of leukocyte since monocytes, macrophages and lymphocytes are able to express this gene.

Much less is known about the transcriptional regulation of TNF-β. It has been shown that TNF-β is transcribed only in lymphocytes and no mRNA can be detected in macrophages (15). In addition, clearly different from TNF-α, unspliced TNF-β mRNA has been detected in the cytoplasm of a murine T-cell line (37). Also it has been demonstrated that accumulation of TNF-β mRNA results primarily from a prolonged half-life of this message contrasting the TNF-α mRNA accumulation, which appears to result primarily from increased transcription (34). However, we detected DH-sites in PBMC but not in the macrophage-like cell culture, supporting the notion that only PBMC produce TNF-β.

Similar to the TNF-α gene the DH-sites of TNF-β were found in a region where putative transcription factor binding sites can be detected (9). Interestingly sites IIIβ and IVβ are not localized

in the promoter region but in the mRNA leader sequence. However, it has been shown in the murine system that the promoter contains a positive regulatory element between bp −293 and +77 and further upstream a negative element for transcription (23). Comparing these results with our analysis we found that the entire site Iβ is situated in the negative element region, whereas IIβ is in the positive element region. The two remaining sites are further downstream of these regions. The relevance of the positions of these sites is not known at the moment and will need further analysis.

In this work we made correlations between the appearance of DH-sites and the transcriptional activity of the porcine TNF genes. Since we started our analysis with nuclei from freshly isolated blood cells, we can be quite sure that we have detected the *in vivo* potential of different cells to produce TNF-α and TNF-β. After establishing the basic pattern of chromatin structure we are now in the position to study the effect of virus infection on the pattern of DH-sites. Also, we are defining at single nucleotide resolution the structure/function relationship in the porcine TNF genes.

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