Molecular Cloning and Expression of an Avian Macrophage Nitric-oxide Synthase cDNA and the Analysis of the Genomic 5′-Flanking Region*

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We report the first nonmammalian inducible nitric-oxide synthase (NOS) cDNA obtained from chicken macrophages. It exhibits an open reading frame encoding 1,136 amino acid residues, predicting a protein of 129,648-Da molecular mass. The deduced NOS protein sequence showed 66.6%, 70.4%, 54.2%, and 48.7% sequence identity to mouse and human inducible NOS and to two constitutive NOSs from rat brain and bovine endothelium. Overall, NOS appears to be a moderately conserved protein. Northern analysis showed that the dothelium. Overall, NOS appears to be a moderately conserved protein. Northern analysis showed that the chicken iNOS mRNA is approximately 4.5 kilobases (kb), a size similar to mammalian inducible NOS. Analysis of 3.2 kb of 5′-flanking sequence of the chicken iNOS gene showed a putative TATA box at 30 base pairs (bp) upstream of the transcription initiation site. The functional importance of the upstream region was determined by transient expression of deletion constructs. An endotoxin regulatory region was located exclusively within 300 bp upstream of the transcription initiation site. This is in contrast to the two distinct sites identified in the mouse macrophage NOS promoter. Transcription factor binding sites such as NF-κB, PEA1, PEA3, and C/EBP were identified. Using a NF-κB inhibitor, we showed that NF-κB is indeed involved in the induction of chicken iNOS gene by lipopolysaccharide. Our results suggest that NF-κB is a common regulatory component in the expression of both mammalian and nonmammalian iNOS genes.

The oxidation of l-arginine (1–3) is now recognized to be an important biochemical pathway in many organisms. One of the products of this reaction, nitric oxide (NO),1 performs many diverse and significant biological functions (4–8). In the nervous system, NO is a novel neurotransmitter (9–11) which is synthesized as needed and not stored in synaptic vesicles. Importantly, nitric oxide does not interact with receptors on the surface of neurons but targets redox centers within neighboring neurons. In the vascular system, NO acts as the endothelium-derived relaxing factor (12, 13) which is a mediator of blood vessel relaxation and blood pressure. It can also inhibit platelet aggregation (14) and adhesion. In the immune system, NO is synthesized by activated macrophages and acts as a cytotoxic and tumoricidal agent (1, 2, 5). NO also mediates important functions in other tissues and organs such as the gastrointestinal tract (15), liver (16), pancreas (17), kidney (18), and the reproductive system (19).

The enzyme, nitric oxide synthase (NOS), which catalyzes the biosynthesis of NO has been purified (20–23), and its cDNA cloned in mammals from the brain (24, 25), endothelium (26–30), macrophage (31, 32), and hepatocyte (33). There are, at least, three genetically distinct types of NOS: type 1 (nNOS), a constitutive form which was initially identified in neurons; type 2 (iNOS), an inducible form from macrophages, and type 3 (eNOS), a constitutive form which was initially identified in endothelium. All isoforms utilize the amino acid arginine, molecular oxygen, and NADPH as substrates and require tetrahydrobiopterin, FAD, and FMN as cofactors (3). The two constitutive forms are activated by and dependent on changes in intracellular calcium (5), whereas the inducible isofrom is calcium independent apparently because calmodulin is a tightly bound subunit of the iNOS (34).

Although there has been considerable research on the biological functions of NO and the regulation of NOS in humans and rodents (35), little is known of NOS in any nonmammalian system. It has been reported that other species are capable of producing NO, such as Limulus polyphemus (35), hemaphagous insects (36), fish (37), and chickens (38). In addition, there is a single report of a primary structure of the constitutive NOS from Drosophila (39) but there are no known NOS sequences from other nonmammalian species. This has not only impeded the understanding the evolution of NOS protein but also made the study of the regulation of NOS at the molecular level in other species impossible. Since chickens do not possess the urea cycle (40) and thus cannot synthesize the substrate of NOS (arginine) directly, the chick represents a unique and potentially important model to study NOS gene expression. In an effort to study NOS regulation and to evaluate the evolution of NOS, we have cloned the first nonmammalian inducible NOS cDNA from a chicken macrophage cell line. In addition, we report the cloning and analysis of chicken iNOS 5′-flanking region. We have identified an upstream region of chicken iNOS gene responsible for LPS stimulation. In this LPS-responsive region, several transcription factor binding elements were identified, but NF-κB was shown to be involved in the induction of chicken iNOS gene expression.
Cloning of Avian Macrophage NOS cDNA and Functional Analysis of 5’ Sequence

MATERIALS AND METHODS

Cell Culture—The chicken macrophage cell line, HD11 (41), was kindly provided by Dr. Dietert (Cornell University, Ithaca). Cells were maintained at 37 °C, 5% CO2 in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% low endotoxin fetal bovine serum (HyClone Laboratories, Logan, UT), penicillin, and streptomycin. LPS (Escherichia coli, serotype 0128:B12) was purchased from Sigma, prepared in DMEM medium at 1 μg/ml, and stored at -80 °C prior to use. All other reagents were purchased from Sigma unless otherwise indicated.

Reverse Transcription-PCR to Generate a Probe for cDNA Library Screening—Highly degenerate oligonucleotides were deduced from conserved amino acid residues between rat neuronal NOS (24) and mouse macrophage NOS (31, 32) sequences for polymerase chain reaction. The reaction was performed by using total RNA from LPS-induced chicken macrophages, HD11. The two degenerate primers were: the sense strand, TTPFVHQEM (ATIACIGCTTCAIAGAIGT), corresponding to amino acids 466-474 in mouse iNOS; the antisense strand, ATETGKS (CGITGTTGCTCITATTG), corresponding to amino acids 538-544 in mouse iNOS. Restriction enzymes, EcoRI and HindIII, recognition sequences were flanked at the 5’ end of the primer sequences for subcloning purpose. A reverse transcription-PCR kit (Perkin-Elmer) was used with the reaction temperature 50 °C for 40 cycles. The PCR product was further subcloned in Bluescript plasmids (Stratagene), sequenced, and used as a probe for cDNA library screening.

cDNA Library Screening—The cDNA library was made commercially (Stratagene) by using 500 μg of total RNA isolated from chicken macrophages stimulated with LPS (100 ng/ml) for 12 h. The HD11 cDNA library was constructed from cDNAs phaged by using random priming and subsequent screening on x-ray films. The PCR product was further subcloned into Bluescript plasmids (Stratagene) and sequenced, and used as a probe for cDNA library screening.

Northern Blot Analysis—Total RNA was isolated using guanidine thiocyanate in a single step method (42), fractionated on a denaturing, 10% formaldehyde, 0.1% SDS at 60°C until radioactivity on membranes was 1% agarose gel, and transferred onto a GeneScreen Plus membrane (DuPont NEN). The blot was hybridized at 42 °C in 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, 100 μg/ml denatured, sonicated salmon sperm DNA, and random priming (32P)CTP-labeled PCR product (1 million cpm/ml) at 42 °C for at least 18 h. Membranes were washed in 0.1% SSC and 0.1% SDS at 60 °C until radioactivity on membranes was below 1,000 cpm and then exposed to x-ray films for at least 8 h before developing. Approximately 1 million independent clones were screened and several dozen positive clones identified. Positive clones were successively spread and screened until pure clones were obtained. Twenty-four positive clones were further rescued to Bluescript plasmids as described by the manufacturer's procedures (Stratagene) for restriction enzyme digestion and DNA sequencing. Manual DNA sequencing was done using Sequence 2.0 kits. (U.S. Biochemical Corp.) and verified using the automated sequencing facility at Cornell University (Applied Biosystems).

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Transient Expression—Chicken macrophages, HD11, were seeded in each well on a 6-well plate 16 to 24 h prior to transfection. The chicken macrophage cell line, HD11 (41), was used for these experiments. Prior to transfection, cells were washed with DMEM without l- or D-arginine (1 mM). Two to three days after transfection, cells were harvested and transferred onto a GeneScreen Plus membrane (DuPont NEN). The blot was hybridized at 42 °C in 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, 100 μg/ml denatured, sonicated salmon sperm DNA, and random priming (32P)CTP-labeled PCR product (1 million cpm/ml) at 42 °C for at least 18 h. Membranes were washed in 0.1% SSC and 0.1% SDS at 60 °C until radioactivity on membranes was below 1,000 cpm and then exposed to x-ray films for at least 8 h before developing. Approximately 1 million independent clones were screened and several dozen positive clones identified. Positive clones were successively spread and screened until pure clones were obtained. Twenty-four positive clones were further rescued to Bluescript plasmids as described by the manufacturer's procedures (Stratagene) for restriction enzyme digestion and DNA sequencing. Manual DNA sequencing was done using Sequence 2.0 kits. (U.S. Biochemical Corp.) and verified using the automated sequencing facility at Cornell University (Applied Biosystems).

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Construction of a Reporter Gene with the Upstream Sequence of iNOS Gene—The primer extension analysis was applied to map the transcriptional initiation site of the chick iNOS gene. The avian myeloblastosis virus reverse transcriptase primed extension system from Promega was used with minor modifications. Total RNA was isolated from chicken macrophages, HD11 cells, stimulated with 100 ng/ml LPS or unstimulated. An oligonucleotide, GTTTGCCAAATCTGCTAG (see Fig. 4), complementary to the 5’ end sequence of the chicken iNOS gene was end-labeled with 32P, and it was used as the primer. The primer annealing temperature was 50 °C for 1 h using 5 μg of total RNA. The reverse transcription was performed in using avian myeloblastosis virus reverse transcriptase at 41 °C for 1 h. The same primer was used in DNA sequencing on a plasmid containing the 5’-flanking region of chick iNOS gene. The sequencing product was run side by side as a molecular weight marker with the primer extension product in a 6% sequencing gel. The gel was dried, transferred to chromatography paper, and exposed to x-ray film. The transcriptional initiation sites of the chicken iNOS gene was obtained by aligning the size of the reverse transcription product with the DNA sequencing result.

Construction of a Reporter Gene with the Upstream Sequence of iNOS Gene—A 3.2-kb fragment of iNOS upstream sequence was cloned into a luciferase reporter gene, pGL2 Basic (Promega). The iNOS 5’-flanking sequence was excised from the plasmid by PvuII and SacI restriction enzymes. The excised fragment was incubated with Klenow enzyme to generate blunt ends for ligation into a luciferase reporter gene. A pGL2 luciferase reporter plasmid was linearized by the Xhod restriction en-
zyme and incubated with Klenow enzyme to generate blunt ends. The linearized and blunt end insert (iNOS 5' end fragment) and vector (pGL2) were incubated together in the presence of the ligation buffer and 1 μl of high concentration T4 ligase (15 units/μl) at 18°C overnight. The ligation mixture was transformed into DH5α E. coli cells and spread on ampicillin agar plates. Six colonies were randomly picked from these plates and their plasmids were isolated. The isolated plasmids were digested individually with restriction enzymes (HindIII and KpnI) to screen the clone containing the iNOS upstream fragment insert. The positive clones were sequenced to determine the orientation of the insert. The clone containing the correct orientation was used to generate deletion clones.

Construction of Deletion Clones—A series of deletion constructs were made from a chimeric plasmid containing the 3.2-kb 5'-upstream region of the iNOS gene ligated in front of a luciferase gene by using a kit, Erase-a-Base system (Promega). The plasmid (20 μg) was digested with MluI and KpnI restriction enzymes to generate 5'- and 3'-protruding ends, respectively. Exonuclease III was used to digest the 5'-protruding end of DNA at 25°C which would digest approximately 90 base pairs per min. An aliquot of DNA was taken every minute to obtain a spectrum of deletion clones. The partial digested DNA was then treated with S1 nuclease and Klenow enzyme to generate blunt ends. The DNA with two blunt ends were ligated together by T4 DNA ligase at room temperature for 1 h. The ligation mixture was transformed into E. coli DH5α competent cells. Plasmid was isolated from randomly picked colonies of deletion clones was estimated by PCR reaction performed by using the primers generated from pGL2 vector close to the polycloning sites. DNA sequencing was used to identify the exact size of the deletion clones.

Transgenic Expression of Deletion Clones—HD11 cells (1.8×106 cells/dish) were seeded in a 100-mm culture dish 24 h before transfection. Cells were kept in DMEM plus 10% fetal bovine serum and antibiotics at 37°C in a 5% CO2 incubator until cells were approximately 60% confluent. HD11 cells were then washed with DMEM buffer without serum and antibiotics three times prior to transfection. For every two 100-mm culture dishes, 4 μg of reporter DNA containing galactosidase gene as a control for transfection efficiency and 4 μg of reporter DNA containing various sizes of iNOS upstream sequence were used in transfection experiments. The two kinds of DNA were mixed well before being distributed into two tubes for transfection. Therefore, each tube contains 4 μg of DNA. LipofectAMINE (30 μl/tube) was then added to each tube. DNA and LipofectAMINE were mixed thoroughly and incubated at room temperature for at least 30 min prior to adding to cell culture dishes. HD11 cells were then incubated with DNA and LipofectAMINE mixture in DMEM without serum and antibiotics. Five hours after transfection, HD11 cells were harvested by scraping with a rubber policeman and pooled from two dishes. Transfected cells were washed twice with DMEM without serum and then reseeded into a 6-well culture dish in the presence or absence of 100 nM LPS. 15 h after LPS treatment, cellular extract was isolated and luciferase and β-galactosidase activities were measured.

RESULTS

Chicken Macrophage NOS Sequence—Oligonucleotide primers (see “Materials and Methods”) designed from the conserved sequences between mouse macrophage NOS (31, 32) and rat brain NOS (24) were used to amplify by reverse transcription-PCR on total RNA from chicken macrophages stimulated with LPS (100 ng/ml). A partial chicken type II NOS (iNOS) cDNA was obtained which was approximately 220 bp and showed 68% sequence identity with mouse iNOS cDNA and no sequence similarity to any other existing proteins in GenBank (data not shown). Thus, the PCR product was used as a probe to screen a chicken macrophage cDNA library. Approximately 1 million independent clones from an unamplified library were screened, and hundreds of positive clones were identified. After secondary and tertiary screening, 24 independent clones were isolated. A clone containing the longest insert was fully sequenced on both strands. It possessed an open reading frame which encoded 1,136 amino acid residues, predicting a protein of 129,648 Da molecular mass. The deduced amino acid sequence of chicken macrophage NOS was aligned with published NOS sequence by using MEGALIGN in DNA STAR, Lasergene (Fig. 1). There were conserved regions in deduced amino acid sequences of chicken iNOS for the binding of iNOS cofactors: heme, calmodulin, FMN, FAD, and NADPH.

Table I shows the percent identity (homology) of NOS protein sequences between different isoforms and species by using “GAP” algorithm in the GCg program. The deduced chicken macrophage NOS protein sequence showed 66.6% (79.1%), 70.4% (83.2%), 54.2% (71.7%), and 48.7% (66.1%) sequence identity (similarity) to mouse iNOS (31, 32), human iNOS (44), rat brain eNOS (24), and bovine endothelium nNOS (30), respectively. Among the three different isoforms of NOS, there is approximately 50% identity within the same species, e.g. identity among iNOS, eNOS, and nNOS in human. However, eNOS or nNOS among different species exhibited 93% identity, whereas the identity of iNOS among different species was about 81%. Thus, it appears that the percent identity among the same isoforms of NOS between species was considerably higher than that of different isoforms within the same species.

Northern Analysis—The Northern blot analysis (Fig. 2) showed the chicken iNOS mRNA possesses a molecular size of approximately 4.5 kb, and the highest accumulation of NOS message at 6–12 h after LPS treatment of HD11 cells. After 12 h, the NOS mRNA abundance decreased and approached the basal levels by 24 h. The chicken macrophage iNOS mRNA molecular mass resembled that of the mouse macrophage iNOS (4.5 kb) and is different from the rat brain constitutive NOS mRNA (9.5 kb) (24).

Transgenic Expression—To investigate whether the clone obtained is functional, transgenic transfection experiments were performed (Fig. 3). Transfection into COS-1 cells of the clone in the sense direction resulted in significant nitrite production, whereas the nitrite production in cells transfected with antisense or vector DNA was undetectable. Furthermore, inhibitors for iNOS, L-NMMA, and aminoxyquanidine (100 μM) were capable of blocking the production of nitrite in COS cells transfected with chicken iNOS cDNA oriented in the sense direction. The inhibition by L-NMMA and aminoxyquanidine could be overcome partially by incubation of cells with excess L-arginine (1 mM) but not d-arginine (1 mM) (Fig. 3).

iNOS 5’-Flanking Sequence—After screening an amplified chicken genomic library, eight positive clones were identified. These eight positive clones belonged to four independent clones as determined by restriction analysis (data not shown). An approach using PCR (see “Materials and Methods”) was used to identify the clone containing the 5’-flanking region of chicken iNOS gene. After identifying the clones which possessed the upstream sequence of iNOS gene, they were subcloned into Bluescript plasmids by four different restriction enzymes. Applying the same PCR approach to identify randomly picked colonies from these four subcloning groups, two colonies were found to possess the upstream sequence of iNOS gene. A total of 3,145 bp of the upstream region of chicken iNOS gene (Fig. 4) was obtained. Comparing the genomic sequences to iNOS cDNA sequences, an intron (97 bp) was identified. This intron was located between +86 and +182 bp.

The mRNA transcription initiation site, designated nucleotide +1, was identified by primer extension analysis using total RNA from LPS-stimulated HD11 cells (Fig. 5). There was no band in the same primer extension analysis by applying total RNA isolated from unstimulated HD11 cells (lane U). A putative TATA box was identified at 30 bp upstream of the transcription initiation site. Comparing the sequence identity of the 300 bases upstream of the transcription start site of chicken iNOS gene to that of human and mouse iNOS showed 39.8% and 35% identity, respectively.

Deletion Analysis—The chicken 5’-flanking sequence was ligated in front of a luciferase gene (a basic form without
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**FIG. 1.** The deduced amino acid sequence of chicken macrophage iNOS (CKiNOS) aligned with the amino acid sequences of murine macrophage iNOS (MUiNOS) (31, 32), human chondrocyte iNOS (HUiNOS) (44), bovine endothelial cNOS (BoeNOS) (28), and rat neuronal cNOS (RAnNOS) (24). Gaps introduced in these sequences to optimize alignments are shown as dots. The identical amino acids are shown as asterisks. Putative cofactor binding sites are drawn in boxes as indicated. CM, calmodulin binding site; Hem, heme binding site; FMN, FMN binding site; F, FAD binding site; N, NADPH binding site. The residues used to design the primers for polymerase chain reaction are shown in bold italic.

promoter and enhancer). Serial deletion clones were generated by exonuclease III digestion. The precise deletion site was determined by DNA sequencing analysis. The deletion clones along with a control gene, β-galactosidase, were transfected into chicken macrophages, HD11. Transfected cells were treated with LPS, and the activities of luciferase and β-galactosidase were measured.

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The protein sequence comparison of NOS from various species: percent identity (homology) shown as percentage

|        | Human iNOS | Mouse iNOS | Human nNOS | Rat nNOS | Human eNOS | Bovine eNOS |
|--------|------------|------------|------------|----------|------------|-------------|
| Chicken iNOS | 70.4 (81.2) | 66.6 (79.1) | 54.6 (71.7) | 54.3 (71.7) | 50.5 (67.5) | 48.6 (66.1) |
| Human iNOS | 53.7 (70.1) | 52.4 (69.6) | 53.7 (70.1) | 51.6 (68.6) | 51.6 (68.6) | 49.9 (67.3) |
| Mouse iNOS | 93.5 (96.9) | 93.5 (96.9) | 93.5 (96.9) | 93.5 (96.9) | 93.5 (96.9) | 93.5 (96.9) |
| Human nNOS | 57.6 (73.8) | 57.6 (73.8) | 57.6 (73.8) | 57.6 (73.8) | 57.6 (73.8) | 57.6 (73.8) |
| Rat nNOS  | 54.1 (70.8) | 51.6 (68.6) | 51.6 (68.6) | 51.6 (68.6) | 51.6 (68.6) | 51.6 (68.6) |
| Human eNOS | 52.4 (69.6) | 51.6 (68.6) | 51.6 (68.6) | 51.6 (68.6) | 51.6 (68.6) | 51.6 (68.6) |

DISCUSSION

We report herein the first nonmammalian inducible NOS cDNA isolated from chicken macrophages. Our data show that NOS is moderately conserved overall, and it is highly conserved with respect to critical cofactor binding sequences. We also identified approximately 3.2 kb of the upstream region of the chicken iNOS gene. From the deletion study (Fig. 6), we show that LPS responsive elements are located exclusively within 300 bp upstream of the transcription initiation site. Within these 300 bp, several consensus DNA binding elements were identified for transcriptional factors which may be regulated by LPS. NF-κB was identified as a potentially important transcription factor since both mouse and human iNOS (31, 32, 44) contain this response element. The results from the deletion and inhibitor studies (Figs. 6 and 7) appear to indicate that NF-κB is indeed a required nuclear factor to activate the chicken iNOS gene expression by LPS.

The chicken iNOS amino acid sequence is approximately 70% identical with mammalian inducible forms of NOS. Assuming divergence of chickens and mammals at approximately 220 million years (47), the rate of evolution can be calculated to be approximately 12 residues/100 million years. This rate of evolution is comparable to that described for histone H1 protein (47) and metallothionein (48), greater than some proteins like histone H4, H3, H2A, and H2B (0.25, 0.30, 1.7, and 1.7 residues/100 million years), cytochrome c (6.7 residues/100 mil-
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Fig. 4. Nucleotide sequence of the chicken iNOS 5’-flanking region. The mRNA initiation site is denoted as nucleotide position +1 which is bold. Intron 1 is enclosed with parentheses located from +86 to +182 (97 nucleotides). The complementary sequence of nucleotides, +52 to +71, was made as a primer (underlined arrow), 20 nucleotides long, for the primer extension experiment. Putative TATA box is located at nucleotide –30 (boxed). The region associated with LPS response was labeled with a vertical line. Potential transcription factor binding sites are underlined and labeled. All the transcription factor binding sites shown are perfect matches with reported consensus sequences.

-1736 TACCCTTTCT TGGTTTCAC CAGCCTCCTA CAGAGGCAA GTAGGGGAGG
-1686 TCTATAGTTA AACATGAAA ATGGAATCA TTTAAATAC TACATAGTT
-1636 CATCCCTTTT TCACTACTAT GTGAATGCT TTCATGCTCT GTGAGAGAAG
-1568 GCTAAGATT TTAAAAAA ATAAGGCA TACAAATTT TACAGCTT
-1536 ACAAACACG AAATCTAG AAATGTTCAAA CTTGTTTTT TCAATGCCCTGA
-1436 ATTAAAAAACAAAATCAG AGTACGTG ACAAGAACAGA AAGAACACAG
-1336 TCTGTCTCAA GAGATTATCC TTTGACGCT CGTACATCT TACATACAA
-1286 GCTATATTCA GAGAACAGT GACATGCTC TACATAGGC
-1236 TACAGAAAC AGTAAATTT CAGGATCCA TAGAAGCAAG
-1186 AAAAAAAATA CTCTTGGCAA CTCTGGGCGA TAGATGTTCAA
-1136 AGAACAAGCG TTTGCTTCTT TCTTGTATAG CTTGTTGCG GATGTTTTCA
-1086 CAAATCACTAT CAAATGTAT AAATACATG CAATATATTT
-1036 AAAACACTAA AAAAGTATGA TATACATG ATACATATAA
-936 TACCAAGGCT TTCTGAGCC AGCACTTGG ATACCAAAGT TACCTTCT
-886 TGCTTGTGAC AGCTCCAGTT TTCTGTCTTT TACATCGGCA GAGAAACTC
-836 AGCTTGTGAC TGTATTCTA TACCCAGTT ATCAAAAAGC AGCTATCTA
-786 GTCTCCCCCT CTTCTGCTCT GTCTCAGTT ACATCATATG
-736 GGTAATACTC TTAAGCTCCA GGAGACGCA TCTGTCCTG CATTAGCACT
-686 GGCTGGTAC CAAAGAGCC CAGAAAAGCA CATCAGGAA CAGAAGG
-636 TGGCAAGATC AGACACATAC AGACGATTG AAGATCTT
-586 CATAAAAAAC AAAAGAAAC AATATATTC GCAATCTAT
-536 AGACAGTACA AATCTGCTCT TGTTAGTTAA ATGGTTATT AATGCTTTA
-486 CTCTGCAATT TCCTGAGTT CTCTGCTCT ATGAAATAC TCTCAGATT
-436 TTAGAAAT CGACGCTTTA GCTCTGAGT CCGTGGCTG CAAAATAGA
-356 GACGGACACC CAGCAGTTAC ATGACGAGT GTTCTCCTG GCTATTTTT
-336 TCCATTTTTC ACCTCGTCTC CAGAGTTCAC TAAAGAAGT CAGAACAGTT
-286 TCTCTTTTCA TACTCATAT CACAAAGAT CAGAAAGAT CAGAAAGAT
-236 AGCATCACTGTCGATGAGA AGGGCTATTT CAGAGAAGAC AAACTCAAC
-186 AGCTTGYTCA CTAATATGATT CGAGCACTGG ATACGGTTG CTCAGATGTT
-136 GCCCTCCCACT TCACCAAGGA AGTGGGAAA TTTTAGGAGG TCTCCCTTGG
-96 ATTCTTACCC ATTTTAGTAT ATCGGCTG TATAGTCC ATGACATGCTT
-56 ATACATATTTGC CACAAAGTTG TTGCTACTG TATTAGGTA TATTAGGTA
-16 GAAGCCAATA CAGTTTGTTG CAGCTGGAGC CAGGAGAGA

Recently, a bidomain structure of rat brain NOS protein was suggested by Sheta et al. (49). The authors demonstrated that nNOS can be isolated in two functionally intact subunits. We were interested to determine whether there is a difference in the conservation within these two domains and therefore examined the chicken macrophage NOS protein sequence by individual domains. The N-terminal domain of chicken iNOS, the sequence before the putative calmodulin binding site, showed 73% to 61% identity to all published NOS sequences and no sequence similarity to other known proteins. The C-terminal domain of chicken iNOS also showed similar sequence identity to all the published NOS sequences (66% to 49%) and high sequence similarity to P450 reductase (50) and ferrodoxin reductase (51). Thus, the two domains of iNOS appear to be equally conserved. Since the putative calmodulin (52) and heme (53) binding sites were highly conserved, this suggests that the N-terminal domain of NOS assumes a function unique to NOS with no similarity to other known enzymes. When aligned with the ferrodoxin reductase sequence (54, 55), the published NOS protein sequences were highly conserved at the NADH and FAD cofactor binding sites (Fig. 1). This reinforces the notion that the catalytic function of the C-terminal domain of NOS is to transfer electrons to heme moieties at the N-terminal domain.

The transcription initiation site of chicken iNOS was identified to be a single start site, G nucleotide, by primer extension. There is a putative TATA box, 30 bp upstream of the transcriptional initiation site which is identical with the mouse (30 bp).
and human (30 bp) iNOS genes (56–58). Furthermore, when total RNA from unstimulated chicken macrophages was used in primer extension analysis, there was no detectable iNOS mRNA. This result confirmed the data seen in the Northern blot analysis and reinforced the observation that the accumulation of chicken macrophage iNOS mRNA is induced by LPS.

The 5' -flanking region, 3,145 bp, of chicken iNOS gene conferred inducibility by LPS. The major finding of the present study is that a 267-bp upstream region contained complete LPS inducibility. The relative luciferase activity of a reporter gene containing 267 bp of the upstream region of the iNOS gene responded fully to LPS stimulation and was not enhanced by the additional 5'-flanking sequence. Thus, the 267 bp upstream of iNOS gene seemed to contain the elements essential for endotoxin-induced transcriptional activation. Moreover, this region can be divided into two subregions: subregion I, −179 bp to −93 bp and subregion II, −267 bp to −179 bp based on the differential activity following LPS activation. The clone containing minimum 5'-flanking sequences, −93 bp to +259 bp, failed to exhibit any promoter activity as stimulated by LPS. With the addition of subregion I in front of this minimum 5'-flanking region, there was a 34-fold increase in LPS-induced luciferase activity as compared to that of the done with minimum 5'-flanking sequences. When subregion II was also present, LPS induced luciferase activity by an additional 5-fold which then totaled a 178-fold increase in LPS-induced luciferase activity when compared to the minimum 5'-flanking sequence. This result suggested that only subregions I and II are required for maximum induction of iNOS gene transcription by endotoxin. Again, this result contrasts observations reported for the mouse macrophage NOS where two distinctly separated regions (−1029 to −913 and −209 to −48) were identified (58).

Analyzing the chicken LPS-sensitive regions revealed several consensus elements for transcription factors which may be responsible for LPS stimulation. Subregion I contains NF-κB (59) and C/EBP consensus binding elements (60). NF-κB has also been shown to exist in the promoters of mouse (56, 58) and human (57) iNOS genes. Moreover, NF-κB has been shown to be a required nuclear factor for the induction of mouse iNOS gene by LPS (46). In the present study, the NF-κB inhibitor, PDTC, was able to block almost completely both nitrite accumulation in culture medium and NOS mRNA accumulation in LPS-stimulated macrophages. This result suggests that NF-κB is also functionally involved in the transcriptional regulation or activation of chicken iNOS gene by LPS. From our data and those of the mouse, we might predict that either the NF-κB consensus element is also functionally significant in human iNOS promoter. If not, then the human iNOS gene represents
a unique iNOS system relative to mouse and avian. Subregion II of the chicken promoter contains PEA1 and PEA3 consensus binding elements (61, 62). These elements have been shown in the enhancer region of polyomavirus, and they are important for polyomavirus DNA replication and RNA transcription (63, 64). PEA1 and PEA3 also appear in the upstream region of collagenase gene (65), and they have been demonstrated to modulate 12-O-tetradecanoylphorbol-13-acetate induction of collagenase gene. However, PEA1 and PEA3 are not present in the promoters of mouse and human iNOS genes. Further studies are needed to characterize the promoter sequence of chicken iNOS in detail to gain a greater understanding of the mechanisms that regulate chicken iNOS gene expression.

The early portion (400 bp) of the 5′-flanking region of human iNOS gene is approximately 67% identical with the mouse iNOS gene (57). Although this upstream sequence of iNOS gene between mouse and human shares high sequence similarity, there is as yet no functional analysis of the 5′-flanking region of human iNOS gene. The 5′-flanking region of chicken iNOS gene was shown in the present study to contain the LPS-responsive region within 300 bp upstream of the transcription initiation site. When comparing the sequence identity between this 300 bp of 5′-flanking sequences of chicken iNOS gene with that of mouse or human iNOS genes, there was a low identity: 35% to mouse iNOS and 39.8% to human iNOS gene. Essentially, the data suggest that there is little sequence identity in 300 bp of 5′-flanking sequences of chicken iNOS gene compared with those of mouse or human iNOS genes. On the other hand, the promoter sequences of the chicken, mouse, and human iNOS genes show: 1) a TATA box located at 30 bp upstream of the transcription initiation site, 2) an NF-κB response element located at approximately 100 bp upstream of the transcription initiation site. Moreover, both chicken and mouse iNOS genes have been demonstrated to possess functional LPS-responsive activity within 300 bp upstream of the transcription initiation site. This indicates that the avian iNOS promoter shares both similarities and differences to the mammalian iNOS promoters. The similarities would suggest that either the 400-bp upstream region of the human iNOS gene is functionally important or that the human gene is uniquely different.

In summary, we have cloned the first nonmammalian iNOS cDNA from a chicken macrophage cell line. The sequence comparison showed that chicken iNOS protein sequence is approximately 70% identical with mammalian iNOS. The rate of evolution of the iNOS protein, overall, appears to be moderate. The molecular size of chicken macrophage iNOS mRNA (4.5 kb) is comparable to the mammalian iNOS. Functional analysis of 3.2 kb of the 5′-flanking sequences of chicken iNOS gene showed a LPS-responsive region located exclusively within 300 bp upstream of the transcription initiation site, a result in marked contrast to that of mouse iNOS gene (56, 58). Also, when comparing the sequences of these 300 bp between chicken iNOS gene to that of mouse (or human) gene, there was low identity (35%). Several consensus sequences for transcription factors were identified: NF-κB, C/EBP, PEA1, and PEA3. Using a NF-κB inhibitor, PDTC, NF-κB was shown to be involved in the LPS-induced iNOS gene expression, a result similar to that of mouse iNOS gene. The similarities and differences observed between the promoters of chicken iNOS gene and those of human and mouse iNOS genes may provide insight into the molecular mechanisms that regulate the expression of iNOS and the factors that influence the expression patterns of iNOS in different species.

Note Added in Proof—deVerla et al. (67) recently reported an analysis of the human iNOS promoter.
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