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Structure, mapping and expression of a growth factor inducible gene encoding a putative nuclear hormonal binding receptor

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We have characterized a growth factor inducible gene, N10, encoding a nuclear protein of 601 amino acids with a significant similarity to members of the steroid and thyroid hormone receptor families. The gene is rapidly but transiently induced by several mitogens. Immunoprecipitation studies show that the N10 protein is transiently expressed after stimulation of quiescent cells, presenting a half-life of ~30 min. The N10 transcription unit is 8 kb in length, split into seven exons. The exon–intron distribution is in general similar to that of other members of the nuclear receptor superfamily, but presents some differences which suggest that N10 belongs to a new family of these molecules. The 5′ flanking region contains one DSE which could explain its immediate response to external stimulus. The N10 gene is located in the [F1-F3] region of mouse chromosome 15.

Key words: G_0–G_1 transition/gene regulation/N10-gene/transcription factor

Introduction

The understanding of the mechanisms controlling eukaryotic cell growth requires a comprehensive knowledge of the earliest biochemical events that occur when a proliferative response is triggered by external signals. In this sense the identification of genes that are specifically induced by mitogens in non-proliferative cells is of great interest. Accordingly, several laboratories have isolated, by cDNA cloning, sets of genes which are immediately induced by serum in quiescent cells (Cochran *et al.*, 1983; Lau and Nathans, 1985, 1987; Lim *et al.*, 1987; Almendral *et al.*, 1988). The early notion that some of the growth factor responsive genes would encode nuclear proteins which could participate in the transactivation of genes required for the progression through G_1 has been strongly supported by the recent findings that the products of several genes induced during the G_0 to G_1 transition are putative or *bona fide* transacting molecules (Chavrier *et al.*, 1988; Christy *et al.*, 1988; Cohen and Curran, 1988; Lemaire *et al.*, 1988; Ryder and Nathans, 1988; Ryder *et al.*, 1988; Ryseck *et al.*, 1988; Sukhatme *et al.*, 1988; Zerial *et al.*, 1989).

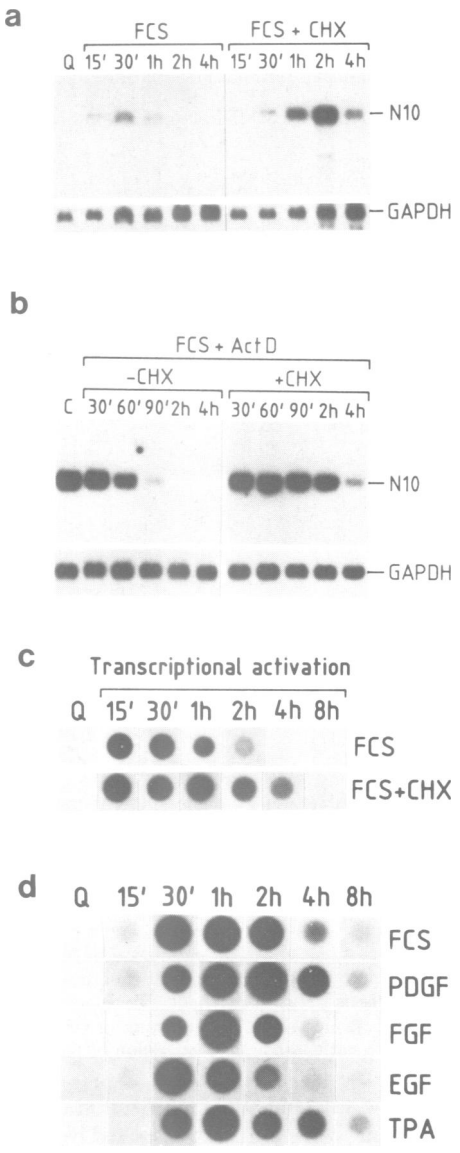


Fig. 1. (**a**) Northern blot analysis of N10 mRNA from serum-stimulated quiescent NIH3T3 cells. Quiescent cells were stimulated with serum for the indicated periods of time in the absence (FCS) or in the presence of cycloheximide (FCS + CHX). The complete nick-translated N10 cDNA was used as a probe. GAPDH (glyceraldehyde-3 phosphate dehydrogenase) was used as a control. (**b**) Determination of the half-life of N10 mRNA. Quiescent cells were stimulated with serum in the presence of cycloheximide for 4 h, followed by actinomycin D treatment in the absence (−CHX) or presence (+CHX) of cycloheximide for the indicated periods of time. The control lane (C) contained mRNA from serum plus cycloheximide-stimulated cells for 4 h. (**c**) Transcriptional activation of N10. Nuclei were isolated from quiescent cells (Q) or cells stimulated with serum alone (FCS) or in the presence of cycloheximide (FCS + CHX) for the indicated periods of time and their transcriptional activity was determined by nuclear run-on assays. The labeled transcripts were hybridized against 1 μg of recombinant pUC19 containing the complete N10 cDNA spotted onto Gene-Screen Plus membrane. (**d**) Induction of N10 mRNA by different mitogens.

		%
grCavCgDnascqHygvrtCegCKgFFkRtvqks..akyiClankdCpvdKrrRnrCqfCRfqKClavGMvk	N10	100
ryCavCnDyasgyHygvwsCegCKaFFkRsiqgh..ndymCpatnqCtidKnrRksCqaCRlrKCyevGMmk	ESTR	56
eqCvvCgDkatgyHyrcitCegCKgFFrRtiqknlhptysCkydgcCvidKitRnqCqlCRfkKCisvGMam	TYHR	54
kpCfvCqDkssgyHygvsaCegCKgFFrRsiqkn..mvytChrdknCiinKvtRnrCqyCRlqKCfevGMsk	RAR	53
kiCliCgDeasgcHygvltCgsCKvFFkRamegq..hnylCagrndCivdKirRknCpaCRlrKCcqaGMvl	PRGR	49
klClvCsDeasgcHygvltCgsCKvFFkRavegq..hnylCagrndCiidKirRknCpaCRyrKClqaGMnl	GCR	49
riCgvCgDratgfHfnamtCegCKgFFrRsmkrk..amftCpfngdCkitKdnRrhCqaCRlkKCvdiGMmk	VDR	47
ktCliCgDeasgcHygaltCgsCKvFFkRaaegk..nkylCasrndCtidKfnRknCpsCRlrKCyeaGMtl	ADR	46
C_I	C_{II}	

Fig. 2. Comparison between the predicted N10 amino acid sequence and the DNA-binding domain of nuclear hormone receptors. Amino acids that are identical in all sequences are written in capital bold letters. The percentage on the right indicates the identity between N10 and the various receptors in that region. C_I and C_{II} indicate the first and second zinc fingers present in these receptors. ESTR, human estrogen receptor; TYHR, human thyroid hormone receptor; RAR, human retinoic acid receptor; PRGR, human progesterone receptor; GCR, human glucocorticoid receptor; VDR, chicken vitamin D receptor; ADR, human androgen receptor.

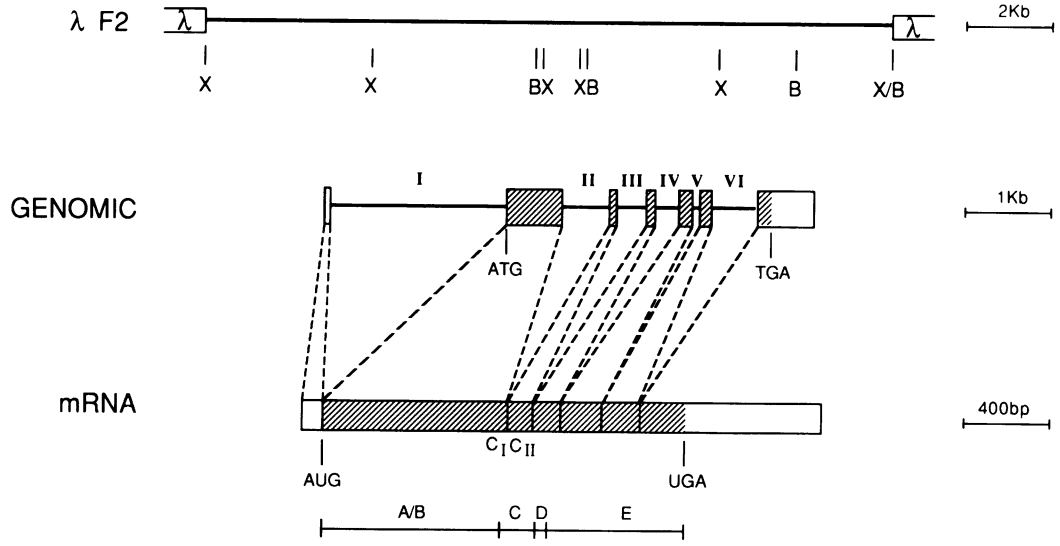


Fig. 3. Genomic structure of N10 gene. A restriction map of ~16.5 kb of the N10 gene covered by the λ genomic clone F2 is presented. An enlargement of the gene is shown underneath. The introns are indicated by roman numerals and the exons by boxes. The shadowed areas correspond to regions encoding the amino acids. The structure of the mRNA is also presented, with the positions of the sites for translation initiation (AUG) and termination (UGA). C_I and C_{II} indicate the position of the zinc fingers. The different regions of the hormone receptors are indicated at the bottom.

Here we present a detailed characterization of a growth factor inducible gene, N10, previously isolated by differential screening (Almendral *et al.*, 1988), whose structure and sequence suggest that it is a nuclear hormone receptor.

Results

Regulation of N10 expression

To determine the mRNA levels of N10 during the G_0 to G_1 transition, RNA was isolated from quiescent NIH 3T3 cells, serum-stimulated for different periods of time and analyzed by Northern blot hybridization using a complete N10 cDNA insert. The 2.5 kb N10 mRNA is very low in quiescent cells but rapidly increases to a significant level 15 min after stimulation, reaching a maximum expression at 30 min, representing an increase of 30-fold with respect to that observed in non-stimulated cells (Figure 1a). Thereafter, the mRNA decreases rapidly, reaching low levels at 2 h and being barely detectable at 4 h after long exposure of the Northern blots. Serum stimulation in the presence of cycloheximide leads to the superinduction of N10 mRNA (Figure 1a). Under these conditions, N10 mRNA reaches the highest level at 2 h, being still very high at 4 h (comparable to the maximum level obtained in the absence of cycloheximide, Figure 1a), suggesting that the mRNA is unstable. To determine the half-life of N10 mRNA, quiescent NIH3T3 cells were serum-stimulated in the presence of cycloheximide for 4 h and subsequently treated with actinomycin D in the absence or presence of cycloheximide. This procedure allows measurement of degradation of accumulated transcripts in the absence of *de novo* transcription. As shown in Figure 1b, the level of N10 mRNA rapidly decreases in the absence of protein synthesis inhibitor, reaching low levels at 2 h and being undetectable at 4 h. The estimated half-life is ~15 min. In the presence of cycloheximide the half-life is

prolonged 4- to 5-fold, being still detectable 4 h after actinomycin D addition.

To study whether the changes in N10 mRNA levels observed after serum stimulation reflect a transcriptional control, nuclei were isolated at various times after serum addition in the absence or presence of cycloheximide and *in vitro* nuclear run-on transcription assays were performed (Figure 1c). A dramatic but transient increase in transcription of the N10 gene is detected, reaching a maximum level 15 min following stimulation, then decreasing rapidly within 2 h and being undetectable thereafter. The presence of cycloheximide prevents the decrease in transcription, prolonging the expression of the gene at least until 4 h. Thus, the cycloheximide superinduction is due to two synergistic effects: prolonged transcription and mRNA stabilization.

Studies with different mitogens clearly demonstrate that the N10 mRNA level is induced by several different pathways. All mitogens tested, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induce N10 mRNA at a similar level, but with slightly different kinetics. The fastest but also more transient induction of N10 mRNA is obtained with EGF. A similar induction has previously been observed for c-*fos* and c-*myc* mRNAs (Bravo *et al.*, 1987). This has been extended to several other immediate early genes (K.Kovary, unpublished observations). It is interesting to note that N10 mRNA can be induced either by protein kinase C dependent pathways (TPA) or by other pathways (EGF). PDGF, which uses more than one pathway, i.e. protein kinase C and Ca^{2+}, has the strongest effect.

Sequence of N10 cDNA and genomic structure

To characterize N10 further, several cDNAs were isolated. The longest cDNA, comprising 2496 nucleotides, contains a large open reading frame of 1803 nucleotides encoding a 601 amino acid protein with a predicted mol. wt of 64 767 (see Figure 4). There is a second possible initiation site 102 nucleotides downstream, which would originate a protein 567 residues long (see below). The 3′ end non-coding region is 643 nucleotides long, contains several in-frame stop codons, and terminates with a stretch of A residues located 17 nucleotides downstream from the consensus polyadenylation signal (Birnstiel *et al.*, 1985). In agreement with the short half-life of N10 mRNA, the 3′ untranslated region contains three times the ATTTA sequence, which is believed to determine the selective degradation of mRNA (Shaw and Kamen, 1986). The 5′ untranslated region of the N10 cDNA is 70 nucleotides long and from the size of the mRNA observed in Northern analysis appears to be practically full length. Primer extension analysis shows that the transcription start site is not precise and is probably located 51–59 nucleotides upstream from the 5′ end of N10 cDNA clone (see Figure 4).

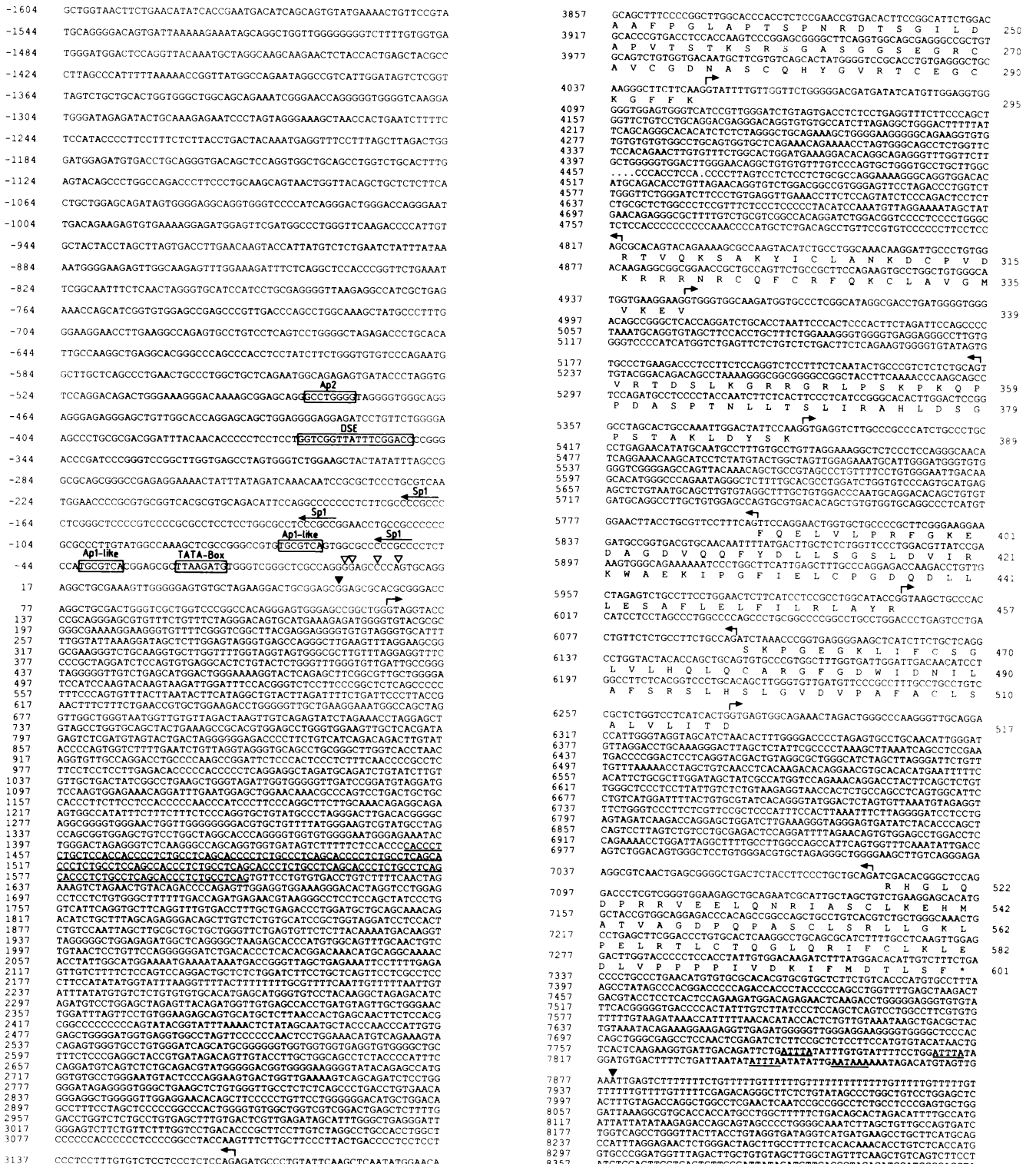
Comparison of the predicted N10 protein sequence with several protein databases has revealed a maximum of 37% similarity (including conserved changes) and 17% identity with some members of the steroid and thyroid nuclear receptor families (for review see Evans, 1988; Green and Chambon, 1988; Shepel and Gorski, 1988; Miesfeld, 1989). The similarity between the N10 protein and members of the superfamily of nuclear ligand binding receptors becomes more evident when comparing the region containing the DNA binding domain of these receptors with amino acids 269–338 of the N10 protein (Figure 2). In this region the N10 protein presents an identity that varies between 46 and 56% with the different receptors, the highest being with the estrogen receptor. N10 contains all amino acids that are identical in all the other receptors, including the eight cysteines that are postulated to be involved in the formation of two zinc fingers, in which four cysteines coordinate with a zinc ion. This strongly suggests that N10 is a new member of the superfamily of nuclear ligand binding receptors. While our work was being carried out, Hazel *et al.* (1988) reported the sequence of a cDNA named nur/77 which is transiently induced by serum and whose sequence is identical to N10 cDNA.

The above observations prompted us to study the genomic structure of the N10 gene in order to determine if its exon–intron distribution would support the notion that it is a hormone receptor. The genomic structure could also help its classification in the appropriate family.

The 2.5 kb N10 cDNA was used to screen a mouse genomic lambda library. Two clones were isolated from $\sim 1 \times 10^6$ plaques screened. One clone λF2, which showed homology to probes from both the 5′ and 3′ part of the cDNA, was analyzed further. Restriction mapping analysis and Southern blotting experiments allowed definition of the region containing the N10 gene. These results are summarized in Figure 3, which presents a restriction map of ~16.5 kb containing the N10 gene. The N10 cDNA clone was shown to be completely included within a region of 9 kb. This part was subcloned from λF2 and analyzed in further detail. Fine restriction mapping indicated the presence of several introns. This was confirmed by the determination of the complete nucleotide sequence of the fragment. Comparison of the genomic DNA and cDNA sequences allowed the precise localization of the intron boundaries (Figure 4). The N10 gene contains seven exons and six introns. The six exons containing the coding part of N10 are distributed in a genomic fragment of ~4.6 kb.

The complete nucleotide sequence of N10 gene is shown in Figure 4. From the TATA box to the poly(A) addition signal AATAAA, the N10 gene is 8000 nucleotides long. The putative 5′-cap-nucleotide is located ~19 nucleotides downstream from the TATA box. As determined by primer extension analysis, the initiation of transcription is not precise (not shown), possibly due to the poorly defined TATA box. Although >3 kb of the 5′ area have been sequenced, only a few *cis*-acting elements within the first 400 bases upstream of the TATA box could be identified by comparison with known consensus DNA motifs. These are three SP1 binding sequences, one AP2 motif, two AP-1-like binding sequences and one DSE-like sequence. We have observed by gel-retardation assays that complexes between members of the JUN and FOS families are able to bind to these AP-1-like sequences found in the N10 gene (data not shown). The role of the different elements in the transcriptional activation or repression of N10 is unknown.

The first exon of the N10 gene contains most of the 5′ untranslated region. The longest intron of the gene separates it from the second exon encoding the first 295 amino acids comprising the putative transcriptional activator domain (A/B) and the first zinc finger (C_I). The A/B regions of the chicken progesterone receptor (Huckaby *et al.*, 1987),


Ap2
DSE
Sp1
Sp1
Ap1-like
Sp1
Ap1-like
TATA-Box

human estrogen receptor (Ponglikitmongkol *et al.*, 1988) and the rat glucocorticoid receptor (Miesfeld *et al.*, 1987) are also encoded in a single exon. However, in contrast, to all other receptors so far described, the first zinc finger domain of N10 is not encoded by a separate exon. Whether the intron has been lost during the evolution of the gene has to be proved. The putative transcriptional activator domain (amino acids 1–252) is very rich in the amino acids serine (45) and proline (35) which amount to 32% of the total residues in this region. It also contains an excess of acidic amino acids (20 Asp + Glu/6 Arg + Lys) as is the case for other transcriptional activator regions (Ptashne, 1988). In contrast, the first zinc finger (amino acids 267–295) contains no prolines, only one serine and has an excess of basic amino acids. The second zinc finger (44 amino acids) is encoded by a third separated exon. Interestingly, the position of the intron is different to that in other members of this superfamily (Green and Chambon, 1988). One position is typical for all the members of the steroid hormone receptor family (Figure 5), the other for the thyroid/retinoic family (Figure 5). The new position of this N10 intron suggests that the evolution of the nuclear ligand binding receptors family is more complicated than previously anticipated (Ponglikitmongkol *et al.*, 1988). The fourth exon encodes probably for all of region D, the 'hinge domain'. The intron between the third and fourth exon is in a conserved position, found in all nuclear receptor genes known so far (Green and Chambon, 1988).

The last three exons encode for the putative ligand domain (region E) and the complete 3′ untranslated region of the mRNA. The amino acid sequence (210 amino acids) of this part is highly charged with a similar amount of basic and acidic residues.

To study if N10 is specifically expressed in some cell types, the level of N10 mRNA was determined in various mouse organs. As shown in Figure 6, N10 mRNA is expressed at its highest level in thymus, followed by testes, heart, brain, spleen and lung. No expression was detected in liver and intestine. The level of N10 mRNA in mouse tissues is at least 20- to 30-fold less than that observed during induction by serum (see Figure 6). It is possible though that only certain cells in the tissue express N10, but at a high level. This needs to be determined by *in situ* hybridization.

Expresssion of N10 protein

Two in-frame methionine codons are present in the N10 sequence that could be used as translation initiation codons giving rise to proteins of 601 or 567 amino acids. To determine which of these codons is preferentially used, the full-length cDNA of N10 and one containing only the second possible translation initation site were cloned in the T7/T3 promoter based vector Bluescript KS (+) and transcribed *in vitro* with T3 polymerase. The RNAs produced were translated in an *in vitro* reticulocyte lysate system. The products were immunoprecipitated with a polyclonal antiserum raised against an N10 fusion protein. Figure 7a shows that the mRNA containing the longest open reading frame preferentially directs the synthesis of a polypeptide migrating as a 70 kd mol. wt molecule. The RNA containing the second translation initiation site gives rise to a smaller polypeptide of ~66 kd. These results confirm the existence of a large open reading frame in N10 mRNA and suggest that the translation initiation site used *in vivo* is preferentially the first methionine codon.

To determine the cellular localization of N10 protein, COS I cells were transfected with a vector containing the complete coding region of N10 under the control of the SV40 late promoter. Expression of N10 was analyzed 48 h later by indirect immunofluorescence. The result shown in Figure 7b clearly demonstrates the nuclear localization of N10 protein. The same result was obtained after incubating the transfected cells for 24 h in the absence of serum, suggesting that N10 protein does not require the natural ligand in order to have a nuclear localization, as has been described for other hormone receptors (Picard and Yamamoto, 1987). Immunofluorescent analysis of serum-stimulated NIH3T3 cells also demonstrated the nuclear localization of N10 protein; however, the intensity of the signal was several-fold lower than the one observed in transfected COS cells (not shown).

To study the kinetics of induction of N10 protein, serum-stimulated cells were labeled for 30 min at different times after serum addition and the synthesis of N10 protein determined by immunoprecipitation followed by gel electrophoresis. As shown in Figure 8, the N10 protein is undetectable in quiescent NIH3T3 cells, but rapidly induced after serum addition. The highest rate of accumulation is 1 h after stimulation and it is barely detectable at 3 h. The estimated size of the protein, ~70–74 kd, suggests that the largest product of N10 is preferentially produced *in vivo*, and its electrophoretic mobility suggests that it is highly modified. Pulse-chase experiments demonstrate that N10 protein has a short half-life of ~30 min (Figure 8).

Chromosomal localization of N10

To determine the chromosomal localization of N10 gene, *in situ* hybridization experiments were carried out using mouse metaphase spreads. A recombinnant pUC19 plasmid containing the complete N10 insert was used as a probe. In the 100 metaphase cells examined after *in situ* hybridization, there were 130 silver grains associated with chromosomes and 60 of these (46%) were located on chromosome 15; the distribution of grains on this chromosome was not random, 70% of them mapped to the [F1-F3] region of chromosome 15 (Figure 9). This strongly suggests that the N10 gene is located in the 15F band of the murine genome. *In situ* hybridization with the human genome demonstrated that N10 maps to the proximal part of the chromosome 12 long arm, i.e. 12q13 (not shown). Interestingly these localizations are very similar to those for the retinoic acid receptor γ (M.-G. Mattéi and P.Chambon, unpublished observations).

Discussion

The early genomic response to growth factors in fibroblasts is of considerable complexity. At least 100 genes are induced during the G_0 to G_1 transition and possibly several genes

Fig. 4. Nucleotide sequence of the N10 gene and of 5′ and 3′ flanking regions. The sites of transcriptional initiation are indicated by open triangles. The first and last nucleotides corresponding to N10 cDNA are indicated by closed triangles. The ATTTA and polyadenylation signal AATAAA are underlined. The intron–exon boundaries are indicated by broken arrows. The TATA box and other elements are boxed or underlined. The DSE is inverted orientation containing a nearly identical sequence to the consensus core DSE sequence [CC (A or $T)_6$GG]. The first C of the consensus is changed to a G. In the first intron the sequence CACCC.TCTGCCTC.AG, which is repeated 10 times (position 1451–1606), is also underlined.

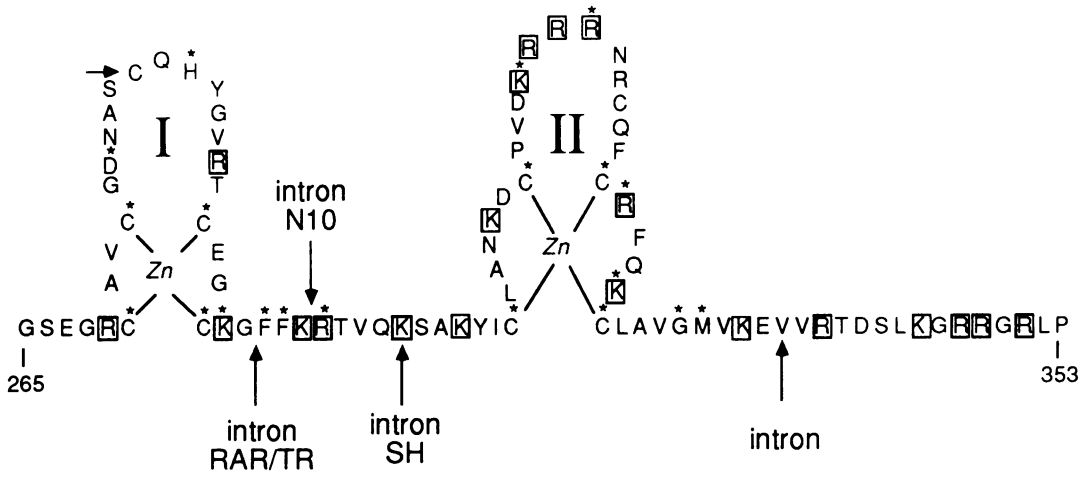


Fig. 5. Intron positions in the zinc finger containing region of N10 and other members of the nuclear hormone receptor superfamily. RAR, retinoic acid receptor; TR, thyroid hormone receptor; SH, steroid hormone receptor. Amino acids which are identical in all receptors are marked with an asterisk. The arrow indicates a cysteine that is only present in N10; all other proteins have glycine in that position. Basic amino acids are boxed (modified from Green and Chambon, 1988).

are simultaneously repressed (Schneider *et al.*, 1988). At present, only a few of the induced genes have been identified. The finding that several of these code for known or probable transcription factors, such as c-*fos* (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Müller *et al.*, 1984), *fos B* (Zerial *et al.*, 1989), *fra-1* (Cohen and Curran, 1988), c-*jun* (Lamph *et al.*, 1988; Ryder and Nathans, 1988; Ryseck *et al.*, 1988), *jun B* (Ryder *et al.*, 1988), Krox-20 (Egr-2; Chavrier *et al.*, 1988; Joseph *et al.*, 1988), and Krox-24 (Zif/268; Egr-1; NGF1-A; Milbrandt, 1987; Christy *et al.*, 1988; Lemaire *et al.*, 1988; Sukhatme *et al.*, 1988) suggests that some of the growth factor inducible genes are involved in the sequential regulation of genes essential for the G_1 progression. Here we have described the characterization of another growth factor inducible gene encoding a nuclear transacting factor. The gene is efficiently induced by several mitogens, including PDGF, FGF, EGF and TPA. The rapid accumulation of N10 mRNA after growth factor addition and the fact that this effect is independent of new protein synthesis suggests that induction of N10 gene expression is a direct consequence of the growth factor−receptor interaction. Recent observations have demonstrated that the homologous gene in rat, NGFI-B, is induced during differentiation of PC12 cells (Milbrandt, 1988), suggesting that its expression is not restricted only to events occuring during initiation of cell proliferation.

Studies on the stability of N10 mRNA demonstrate that its half-life is 10−15 min and that it is prolonged by cycloheximide. The short half-life of N10 mRNA is consistent with the high percentage of A and T in the 3′ untranslated region and the presence of several copies of the ATTTA sequence. These characteristics have been found to be common to a number of unstable mRNAs (Shaw and Kamen, 1986).

The N10 gene codes for a nuclear protein which is rapidly induced after stimulation. The half-life of the protein is very short, ~30 min. Due to its transient expression and short half-life the protein is present in the cell after stimulation for only a few hours.

The predicted sequence of the N10 protein presents a significant similarity with members of the superfamily of hormone nuclear receptors. These share a similar structure,

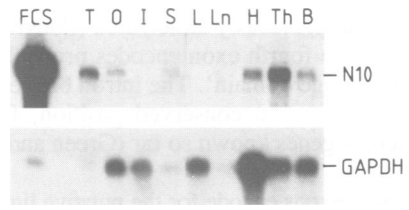


Fig. 6. Expression of N10 mRNA in mouse tissues. Two micrograms of poly(A)$^+$ mRNA were applied in each case. T, testis; O, ovary; I, intestine; S, spleen, L, liver; Ln, lung; H, heart; Th, thymus; B, brain. As control, 0.2 μg of poly(A)$^+$ mRNA from serum-stimulated cells for 4 h in the presence of cycloheximide have been included. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

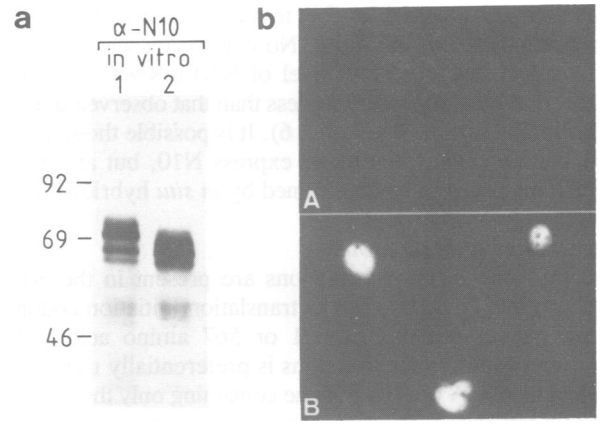


Fig. 7. *In vitro* expression and cellular localization of N10 protein. (**a**) *In vitro* translation products of N10 mRNA using a rabbit reticulocyte system were immunoprecipitated using a specific rabbit antibody (α-N10) raised against the complete N10 protein. **Lane 1** shows the products obtained with the complete open reading frame and **lane 2** when the first methionine has been eliminated. (**b**) Immunofluorescence analysis of N10 protein expressed in COS I cells transfected with a SV40 late replacement vector without (**A**) or with the complete coding region of N10 (**B**).

suggesting that the genes encoding them have evolved from a common ancestral gene. Comparison of the different receptors reveals several regions of varying homology, of

suggesting that the genes encoding them have evolved from a common ancestral gene. Comparison of the different receptors reveals several regions of varying homology, of which region C, that corresponds to the DNA binding domain, is the most conserved. N10 protein contains a region that is equivalent to region C, containing all the conserved amino acids observed in all hormone nuclear receptors. The first half of the N10 region C (C_I) contains 10 conserved amino acids including four cysteines involved in the coordination of a zinc ion to form a zinc finger, and several hydrophobic amino acids. The second half of the N10 region C (C_{II}) contains 11 conserved amino acids including the four cysteines that would participate in the formation of the second zinc finger. This half is very basic, it contains 11 basic amino acids against two acidic amino acids, a property found in all other nuclear receptors.

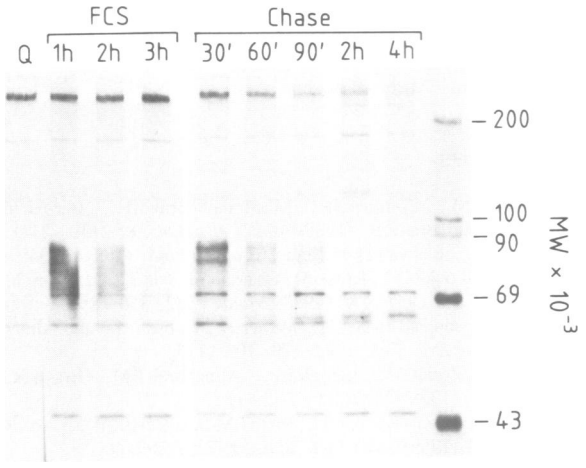


Fig. 8. Induction and stability of N10 protein. induction of N10 protein was determined by immunoprecipitation of extracts from cells stimulated for the indicated times (**left panel**). Cells labeled for 30 min with [^{35}S]methionine were then incubated in medium containing 10 times the normal amount of methionine for the indicated times, lysed and immunoprecipitated with anti-N10 antibody (**right panel**).

The structure of the N10 gene reveals that the corresponding A/B region of the N10 protein is encoded completely in a single exon (exon 2) as for several of the nuclear ligand receptor molecules. The C_I region of N10, in contrast to the other nuclear receptors, is encoded together with the A/B region. Another point of interest in the structure of the N10 gene is the position of intron 2 which separates the exon 2 containing the first zinc finger from the second zinc finger (region C_{II}). The position of this intron is different to that found in the thyroid hormone (Zahraoui and Cuny, 1987), retinoic acid (Dejean *et al.*, 1986; Brand *et al.*, 1988), progesterone (Jeltsch *et al.*, 1986; Huckaby *et al.*, 1987), and estrogen (Ponglikitmongkol *et al.*, 1988) receptors, suggesting that the N10 gene is a member of a novel family of receptors. The position of intron 4 is identical to that described for all other nuclear receptors (Green and Chambon, 1988).

Sequences similar to the DSE found in the 5′ flanking region of the N10 gene have been described for other immediately early genes, such as c-*fos*, Krox-20 and Krox-24, and demonstrated to be functional in serum stimulation assays (Treisman, 1985; Lemaire *et al.*, 1988; Chavrier *et al.*, 1989). Although we have not demonstrated that the DSE present in N10 is functional, it appears from the above observations that this sequence is a common element in the regulation of a number of immediate early genes.

Materials and methods

Cell culture and RNA extraction

NIH3T3 cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U/ml

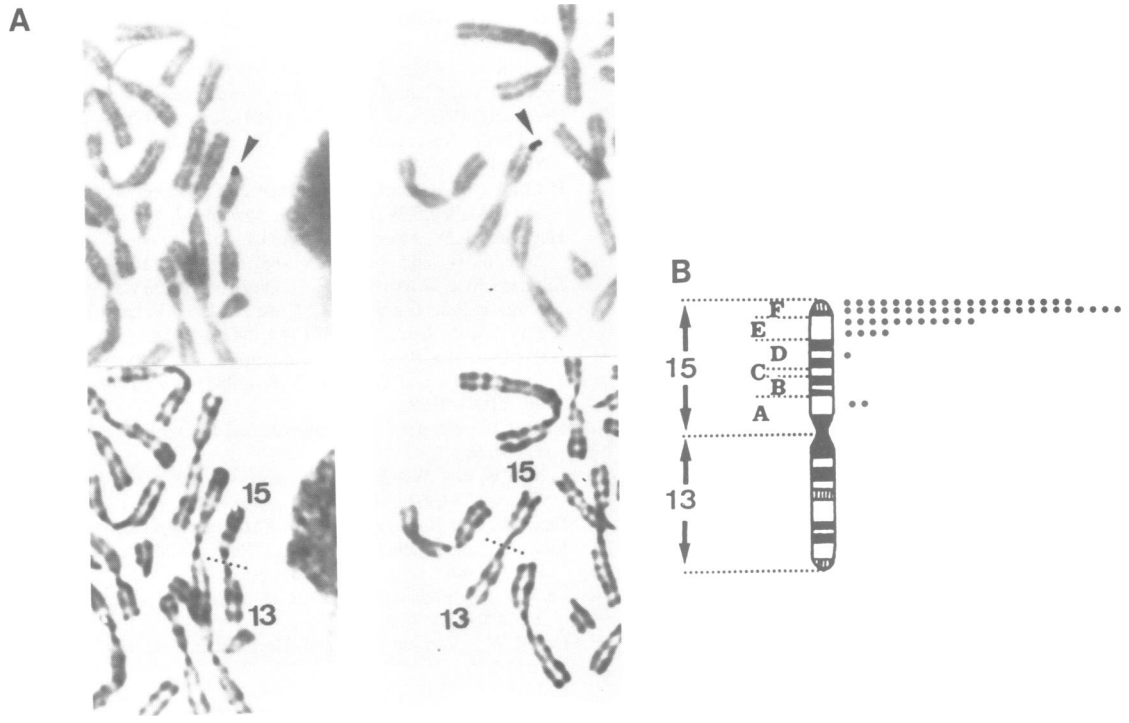


Fig. 9. Localization of the N10 gene to mouse chromosome 15 by *in situ* hybridization. (**A**) Two partial WMP mouse metaphases, showing the specific site of hybridization to chromosome 15. Top, arrowheads indicate silver grains on Giemsa-stained chromosomes, after autoradiography. Bottom, chromosomes with silver grains were subsequently identified by R-banding. (**B**) Diagram of WMP mouse Rb (13;15) chromosome, indicating the distribution of labeled sites.

penicillin, 50 μg/ml streptomycin). Confluent cells were made quiescent by incubating them for 48 h in medium containing 1% FCS. When used, cycloheximide was added at 10 μg/ml and actinomycin D at 1 μg/ml. Total RNA was prepared from cells and tissues using the guanidine hydrochloride procedure (Chirgwin *et al.*, 1979). To obtain poly(A)$^+$ RNA, total RNA was dotted onto messenger-activated paper (mAp; Orgenics Ltd), washed twice for 15 min in buffer, and then washed with 70% ethanol for 10 min. After the messenger-activated paper dried, the poly(A)$^+$ RNA was released by incubation in water at 70°C for 5 min. For Nothern blot analysis, RNA was separated on 1% agarose gels containing 6% formaldehyde (Thomas, 1980) and blotted onto Gene-Screen Plus (New England Nuclear). Purified inserts were ^{32}P-labeled by nick translation (Rigby *et al.*, 1977) to a specific activity of $1-5 \times 10^8$ c.p.m./μg. Hybridization was carried out in 50% formamide, 0.5% SDS, 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate) and 5 × Denhardt's solution at 42°C for 40 h. Filters were extensively washed in 0.1 × SSC containing 0.5% SDS at 60°C.

Nuclear run-on transcription assay

Nuclei from NIH3T3 cells were isolated and run-on experiments were performed as described (Almendral *et al.*, 1988).

In vitro and in vivo translation

In vitro transcription was carried out using the Boehringer transcription kit. For the expression of the longer ORF, the template used was plasmid Bluescript KS (+)-N10 lacking the last 340 nucleotides of N10 cDNA. To obtain a transcript initiating in the second methionine the plasmid Bluescript KS (+)-N10 was digested with *Nco*I (position 172 of N10 cDNA) and *Sal*I (from the polylinker), and Klenow treated and ligated. Both plasmids were linearized with *Bam*HI and transcribed using the T3 polymerase. *In vitro* translation in a rabbit reticulocyte lysate system was done with the BRL translation kit.

For *in vivo* expression, COS I cells were transfected using the DEAE–dextran procedure with pSV51L vector (Huylebroeck *et al.*, 1988) without or containing the complete coding sequence of N10 under the control of the SV40 late promoter. Two days after transfection cells were methanol fixed and incubated with rabbit antiserum raised against the complete sequence of N10, followed by rhodamine-labeled goat anti-rabbit immunoglobulin.

Genomic library screening and Southern blotting

The genomic library was generated by cloning fragments from a partial digestion of mouse DNA with *Sau*3a into λ Dash vector (Stratagene). A total of 1×10^6 phage plaques were screened as previously described (Maniatis *et al.*, 1982). Hybridization and washing conditions were identical to those used for Northern blots. The complete N10 cDNA nick-translated to a specific activity of 5×10^7 c.p.m./μg was used as a probe. For Southern blotting experiments, restriction fragments from phage DNA were separated on 1.2% agarose gels and transferred to a Gene-Screen Plus membrane (New England Nuclear) according to Southern (1975). Hybridization and washing were carried out as described above.

DNA sequencing and sequence analysis

A 9.5 kb DNA fragment isolated from λF2 and containing the complete N10 gene including the 5′ and 3′ flanking sequences was subcloned into the site of pUC19. Various restriction fragments derived from this plasmid were subsequently cloned into M13 derived vectors and nested deletions were performed using a Pharmacia kit. Fragments corresponding to a series of ~250 bp deletions were used for sequencing. Single-stranded DNA was obtained (Messing, 1983) and nucleotide sequence of both strands of each clone was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using the T7 DNA sequencing kit (Pharmacia). Nucleotide and amino acid sequence analyses were carried out using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package, Version 6 (Devereux *et al.*, 1984).

Antisera

To raise antisera against N10, a fusion protein was prepared by recloning most of the cDNA (*Sma*I site, position 411 in Figure 2 until end) in pEx 34a (Strebel *et al.*, 1986) and expressed in *Escherichia coli* K537. After purification, the fusion protein was injected into rabbits using routine protocols. The specificity of the antibody was tested by immunoprecipitation and immunoblotting.

Chromosome spreads preparation

In situ hybridization experiments were carried out using metaphase spreads from a WMP male mouse, in which all the autosomes except 19 were in the form of metacentric Robertsonian translocations. Concanavalin A-stimulated lymphocytes were cultured at 37°C for 72 h with 5-bromodeoxyuridine added for the final 6 h of culture (60 μM/ml of medium), to ensure a chromosomal R-banding of good quality. The N10 clone containing an insert of 2000 bp in pUC19 was tritium labeled by nick-translation to a specific activity of 2×10^8 d.p.m./μg. The radiolabeled probe was hybridized to metaphase spreads at final concentration of 25 ng/ml of hybridization solution as previously described (Mattéi *et al.*, 1985). After coating with nuclear track emulsion (Kodak NTB$_2$), the slides were exposed for 15 days at +4°C, then developed. To avoid any slipping of silver grains during the banding procedure, chromosome spreads were first stained with buffered Giemsa solution and the metaphases photographed. R-banding was then performed by the fluorochrome–photolysis–Giemsa (FPG) method and metaphases rephotographed before analysis.

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