# Regulation of the multiple promoters of the human aldolase A gene: response of its two ubiquitous promoters to agents promoting cell proliferation

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## ABSTRACT

**The human aldolase A gene is transcribed from three distinct promoters, the two ubiquitous promoters $P_N$ and $P_H$ and the muscle specific promoter $P_M$. In the present study, we investigate further aldolase A mRNA structure and expression. We demonstrate that the upstream N-type exon is, in fact, extremely heterogeneous. RNAse H mapping experiments permit quantification of relative abundancy of N, M, and H type mRNAs and show that the level of transcripts containing the downstream H-type exon is at least 30 times higher than that of those containing N exon, in all tissues tested. Aldolase A level is up-regulated in proliferating cells. Here we show that both N and H type mRNAs, although barely detectable in normal liver, are highly expressed in human hepatomas biopsies. Furthermore, in human lymphocytes, N-type mRNA level is enhanced by serum treatment, while in cultured Hep G2 cells, both N-type and H-type mRNA levels are increased by serum and by the tumor promoting agent PMA. Using CAT constructs in transfection experiments, we demonstrate that the H exon plus its upstream region can function autonomously: the 420 base pairs upstream of the H exon are sufficient to confer to promoter $P_H$ an efficiency comparable that of the complete SV40 early promoter and enhancer in two cell lines.**

## INTRODUCTION

Differential expression of a gene can be achieved during evolution through the emergence of multiple promoter regions each endowed with a specific capacity to respond to particular cellular or metabolic conditions. Several examples of genes using alternative promoters are now documented (1), among which the human (2,3), rat (4), and mouse (5) aldolase A gene.

Glycolytic enzyme aldolase A is found in a variety of tissues, either alone in muscle, where it is particularly active, or associated with one of the other two isoforms (aldolase B in intestine and kidney, aldolase C in brain, spleen and blood cells (6). Moreover, it is quite abundant in fetal tissues and, while normal adult liver contains almost no aldolase A activity, this isoenzymatic form can be found reexpressed in hepatomas (7, 8). For this reason, aldolase A can also be considered as a marker of proliferating tissues.

In humans, three types of mRNAs encoding this enzyme are produced from three alternative promoter regions (2). These three types of mRNA differ in their 5′ untranslated regions that consist of specific alternative exons, spliced to a common coding region. The most upstream promoter, $P_N$, and the most downstream one, $P_H$, govern the synthesis of ubiquitous heterogeneous mRNA species, while the central promoter $P_M$ controls the expression of a shorter, skeletal muscle-specific mRNA.

In this paper we refine the structure of mRNA species produced from the N promoter region and characterize the expression of the three types of mRNAs in normal tissues and hepatomas.

The expression of a great variety of genes can be induced when resting cells are stimulated either by serum or by other mitogenic agents, such as phorbol esters. Moreover, serum treament increases the level of several glycolytic enzymes quite before the onset of DNA synthesis (9, 10, 11). This has led us to examine whether, in cultured cells, the level of N or H aldolase A mRNAs was affected by serum or phorbol 12-myristate 13-acetate (PMA). We show that, in human lymphocytes, N-type mRNA level is enhanced by serum treatment, and that, in hepatoma HepG2 cells, both N and H types mRNA production is induced by growth promoting agents.

Finally, we demonstrate that the $P_H$ promoter of the aldolase A gene is as efficient as the SV40 early promoter and enhancer, in transient transfection experiments using the reporter CAT (chloramphenicol acetyl transferase) gene.

## MATERIAL AND METHODS

### RNA analysis

Total RNAs were isolated, electrophorized and analysed by Northern blot as previously reported (12,13)

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For RNAse H mapping, 20 μg of RNAs were coprecipitated with an excess of single-stranded oligonucleotide $0_{25}$ : 5′ CAG CTC CTT CTT CTG CTC CGG GGTC. The pellet was suspended in 10 μl of RNAse H buffer (×1 : 20 mM Tris pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM DTT, 5% sucrose). After 10 minutes at 70°C, hybridization was performed for 30 minutes at 37°C. Then 40 μl of RNAse H buffer containing 7U of RNAse H (Amersham) were added and digestion was carried out for 45 minutes. The products of digestion were loaded on a polyacrylamide-urea gel after ethanol precipitation. The gel was washed twice in 7% formaldehyde, 0.1×TBE and RNA was transfered passively onto a Nylon membrane.

Amount of specific mRNA was measured by densitometry scanning of the autoradiograms of Northern or RNAse H experiments using a Shimadzu densitometer.

$S_1$ mapping and primer extension analysis reactions were done essentially as described (2) except that for $S_1$ mapping, end-labelled or quasi-end labelled probes were hybridized in 10 μl of 40 mM Pipes (pH 6.5), 400 mM NaCl, 1 mM EDTA either at 25°C with 50% formamide for mapping of exon N1, or at 42°C with 80% formamide for mapping of exon N2 ( buffer A).

5′ end labelling was performed by kination of oligonucleotides using T4 polynucleotide kinase. Quasi-end labelling was obtained after extension of an oligonucleotide hybridized to a single stranded M13 template first in the presence of a labelled nucleotide, then in a second step in the presence of the four unlabelled nucleotides in excess.

Primer extension analysis was done by hybridization of 20 μg of total RNAs with end-labelled oligonucleotide $N_{17}$ in ×2,5 reverse transcriptase buffer at 42°C, or quasi-end labelled 79 and 107 bp primers in 10 μl of buffer A at 42°C. Following hybridization, ethanol precipitation was carried out for the 79 bp and 107 bp primers and the pellet was dissolved in 100 μl of reverse transcriptase reaction mix. The 79 bp and the 107 bp primers were obtained by extension of oligonucleotide $N_{17}$ into a $M_{13}$ single-stranded recombinant vector, and then cutting by Ppum 1 or Hinf 1 restriction enzymes, respectively.

The same procedure was carried out to generate $S_1$ mapping analysis probes using specific primers located at 3′ end of $N_1$ or $N_2$ exons. The $N_1$ primer was 5′ end labelled by kination and $S_1$ probe was obtained after elongation of this primer.The $N_2$ primer was not labelled, and labelling was incorporated by the quasi-end method. Both S1 probes were generated by cutting with Eco $R_1$ restriction enzyme.

### Plasmid construction

Po-CAT is a recombinant pEMBL19$^+$ plasmid in which the chloramphenicol acetyl transferase CAT coding sequence and the 3′ SV40 poly A site of PSV2-CAT (sequence from nucleotide 3386 to 5018 as described by Gorman et al (14) have been inserted in an Eco $R_1$ cut, blunt-ended plasmid (15). N and H-CAT plasmids are derived from PoCAT. N-CAT construct includes the sequence we have published (2) from position 0 to 1800 (Asp 700 site in exon $N_2$) inserted into a $BamH_1$ $Sma_1$ Po-CAT. H-CAT contains fragment $Pst_1$ (position 2693) to $Fsp_1$ (position 3454) site inserted in a $Pst_1-Sma_1$ Po-CAT. These constructs are represented in Fig. 8b.

For transfection analysis, plasmids were purified on two successive caesium chloride gradients.

### Cell culture

Hep G2 and LTK$^-$ cells were grown in Dulbelcco's modified Eagle's medium (DMEM) with 10% of fetal calf serum (FCS).

Induction treatment was done after 48 hours of starvation (without FCS), by adding medium either with 1 μM PMA from Sigma, 10% fetal calf serum, 5μM calcium inophore A23187 from Sigma, or 10 μM forskolin.

Lymphocytes were stimulated as described in (16).

### Transfection experiments

Transfection were carried out by the calcium phosphate coprecipitation technique described elsewhere (17). For all cells, the medium was changed 3 to 4 hours before transfection. Generally 10 μg of plasmid CAT-DNA was co-precipitated with 400 ng of an internal control plasmid, pRSV/L which contains the coding sequence of a firefly luciferase cDNA (18). CAT activities were essayed as in (14), and luciferase activity as described in (18). Two different CAT-plasmid preparations were systematically assayed.

## RESULTS

### N promoter region possesses particularly heterogeneous transcription start sites

We have examined extensively the transcription start sites in the N region using three approaches. Those are summarized in Fig.1: the intron-exon structure of the 5′ end of the aldolase A gene is represented, up to scale, and below are positionned the DNA fragments used for S1 nuclease mapping, primer extension or RNAse H mapping analysis.

Nuclease mapping analysis was performed with a single stranded end-labelled genomic probe starting at the 3′ end of exon N1 and overlapping that exon (Fig. 2A).

Four fragments of 64, 58, 38 and 33 nucleotides were prevailingly protected, in muscle (lane1) and Hep G2 cells RNA

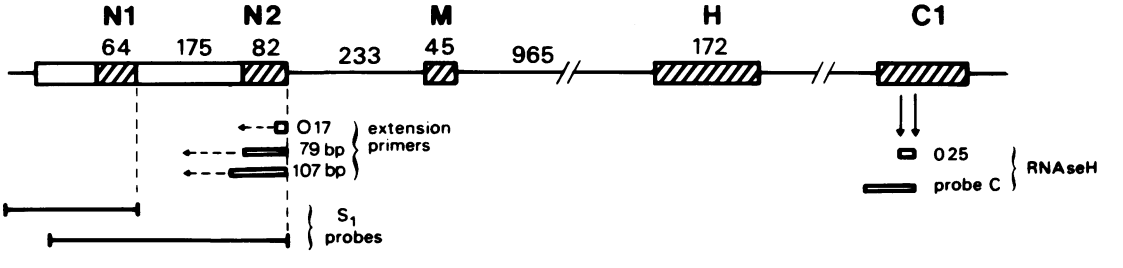


**Fig. 1:** Schematic diagram of the 5′ region of the aldolase A gene. $N_1$, $N_2$, M, H, and $C_1$ exons as well as introns described previously (2) are represented up to scale with their size given in base-pairs. Three types of experiments were used to characterize transcription start sites with respect to the gene. The DNA fragments used for $S_1$ mapping, primer extension and RNAse H mapping are positionned in the lower part of the figure.

(lane 2), all corresponding to presumptive start sites. The abundant 64 nt species corresponded to the size of exon N1 as defined previously from sequence comparisons (2).

All four main transcription initiation sites were confirmed by primer extension analysis. These experiments were performed with different types of primers: either an oligonucleotide complementary to 3′ end of N2 exon (Fig.2C), or DNA fragments encompassing part of exon N2 (Fig. 2D and 2E). Both types of experiments yielded extension products consisting of two doublets (148, 137, 119 and 114 nt) that fit with the start sites in N1 exon, as determined by the S1 nuclease protection experiments, plus, in muscle tissues, a group of larger fragments (Fig. 2C, 2D and 2E). RNAse H mapping experiments, described further on, also confirmed the existence of the main four start sites in the 'N1 exon'.

The existence of much longer, muscle-specific, extension products, the two stronger ones being at 235 and 197 nt (Fig. 2 C and D), did not correlate with the results of the S1 mapping using a probe overlapping exon N1 shown by Fig. 2A. We thus hypothesized that, at least in muscle, RNA transcription initiation could also occur between the presumptive N1 and N2 exons ; this was indeed confirmed by a S1 nuclease protection test using as a probe a single stranded end-labelled genomic DNA fragment encompassing exon N2 and 200 bases upstream of it, i.e. intron *a*, exon N1 and 50 bases of the 5′ flanking region (Fig. 2B), and by primer extension analysis using as primer a DNA fragment encompassing the totality of exon N2 and 25 nt of intron *a* (Fig 2 E).

These additional initiation sites, clustered in the so-called intron *a* (the main being located at 115 nt upstream of exon N2, as represented in Fig. 3) are used in skeletal (Fig. 2B, lane 1) and cardiac muscle (Fig. 2B, lane 4) where they account for less than 10% of N type aldolase A mRNA. They were not found in other tissues (Fig. 2B, lane 2 and results not shown).

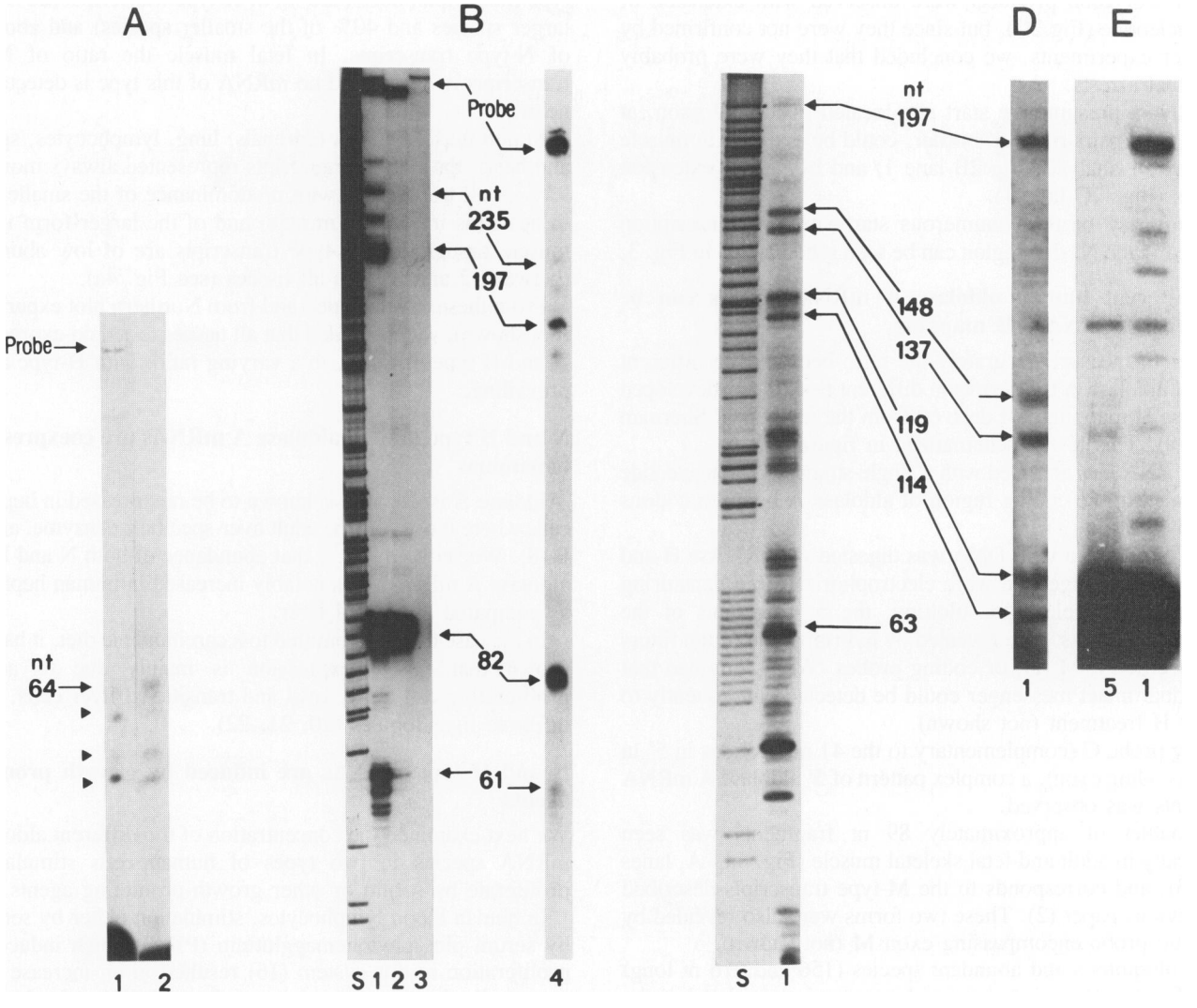


**Fig. 2:** $S_1$ mapping and primer extension analysis of the N region. The single stranded probes used for the experiments of $S_1$ nuclease protection as well as the primers used for the experiments of primer extensions are represented in Fig. 1. Sizes of the protected (A, B) or extended (C, D and E) fragments are given in base pairs; they are calculated by comparison with sequencing reactions deposited onto the gel as standards (lanes S in parts B and C). **A**: Protected fragments after hybridization of a 180 base-pair, 5′ end labelled $N_1$ probe with adult skeletal muscle (lane 1) or cultured Hep G2 cells (lane 2) RNAs. **B**: Protected fragments after hybridization of a 350 base pair, 5′ quasi-end labelled $N_2$ probe with adult skeletal muscle RNAs (1), lymphocyte RNAs (2), rat muscle RNAs (3) used as a negative control, or adult heart RNAs (4). **C**: Primer extension of the 5′ end labelled $O_{17}$ oligonucleotide hybridized with skeletal muscle RNAs. **D**: Primer extension of the 5′ quasi-end labelled 79 bp primer hybridized with skeletal muscle RNAs. **E**: Primer extension of the 5′ quasi-end labelled 107 bp primer hybridized with skeletal muscle RNAs (1), or with rat muscle RNAs (5) used as a negative control. In **C**, **D** and **E**, the arrows indicate those extended fragments which correspond to protected fragments in A and B. We assume that the other bands could correspond to premature arrests of the primer extensions.

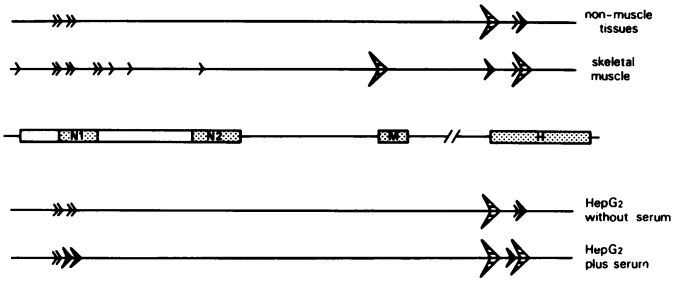


**Fig. 3:** Scheme of the different start sites of transcription of the human aldolase A gene in function of the tissue.The exons are represented as boxes. The delineation of 'exons' and 'introns' in the N region is complex, due to the multiple initiation sites. The '$N_1$' and '$N_2$' exons, first determined from sequence comparisons (2) are stippled as well as the M and H exons. The different transcription start sites are represented by arrowtips whose size is semi-quantitatively related to the abundance of the corresponding transcripts.

Other extension products were observed with extension of oligonucleotide (Fig.2C), but since they were not confirmed by the other experiments, we concluded that they were probably artefactual arrests.

Finally, a presumptive start site located inside N2 exon, at 61−63 base pairs of its 5′ border, could be detected in muscle tissue by S1 analysis (Fig 2B lane 1) and by primer extension analysis (Fig. 2C lane 1).

A summary of these numerous start sites of transcription scattered in the N1-N2 region can be seen schematized in Fig. 3.

## The different human aldolase A mRNA species can be quantified by RNAse H mapping

In order to measure accurately the ratio between the different types of aldolase A transcripts in different tissues, we developed a RNAse H mapping test derived from the method of Sherman et al (19). This test is schematized in figure 4b.

Total RNA was annealed with a single-stranded oligonucleotide $O_{25}$ located in the coding region of aldolase A between codons 8 to 16 (see Fig. 1).

RNA hybridized with DNA was digested with RNAse H and the products of digestion were electrophorized in a denaturing polyacrylamide gel. After blotting, the 5′ fragments of the aldolase A mRNAs were revealed by hybridization of the filters with specific N, M, H, or coding probes. We ascertained that no residual intact messenger could be detected consequently to RNAse H treatment (not shown).

Using probe C (complementary to the 41 nucleotides in 5′ in the first coding exon), a complex pattern of 5′ aldolase A mRNA fragments was observed.

A doublet of approximately 89 nt fragments was seen specifically in adult and fetal skeletal muscle (Fig. 4a−A, lanes 2 and 3), and corresponds to the M-type transcripts described in a previous paper (2). These two forms were also revealed by a specific probe encompassing exon M (not shown).

Two ubiquitous and abundant species (156 and 216 nt long) fit the fragments expected from the transcripts initiated at the downstream and upstream start sites of exon H, as detected earlier by S1 nuclease mapping (2). The smaller species was predominant in skeletal (Fig. 4a−A lanes 2 and 3) and cardiac muscles (lanes 5 and 6), while the larger species was predominant in all the non-muscle tissues (Fig. 4a−D, lanes 4 and 7 to 17).

Hybridization with a H-exon specific probe confirmed that these two species contained exon H, as did a third, faintly expressed, intermediate one, (not shown).

In addition to the M (in muscle) and H-type fragments, several weaker, ubiquitous components of size ranging between 160 and 190 nt were visualized with the coding probe. Those were tentatively identified as N-type fragments, 190, 184, 164 and 158 nt long, corresponding to the four initiation sites in N1 exon, and 203 nt long, corresponding to the upstream initiation site. This was indeed confirmed through the use of N1 (Fig. 4a−B) and N2 (Fig. 4a−C) specific probes, on muscle RNAs. A 241 nt, muscle-specific fragment that hybridized with the N2 probe (Fig.4a−C) and not with the N1 probe (Fig. 5B), fits with the major transcript initiated in intron *a* between exons N1 and N2, as defined previously by S1 nuclease and primer extension. This 241 nt long fragment hybridized with a probe overlapping intron *a* (not shown).

Steady state level of the various types of aldolase A mRNAs could be quantified by densitometry scanning of these experiments. In skeletal muscle, we estimated that steady-state aldolase A mRNAs level was composed of 35% (±5%) of M-type transcripts, 60% (±5%) of H-type transcripts (20% of the larger species and 40% of the smaller species) and about 5% of N-type transcripts. In fetal muscle the ratio of M-type transcripts is lower, and no mRNA of this type is detectable in heart.

In non-muscle tissues (adrenals, lung, lymphocytes, spleen), and heart, the H-type transcripts represented always more than 95% of all transcripts, with predominance of the smaller form in heart (as in skeletal muscle) and of the larger form in non-muscle tissues. The N-type transcripts are of low abundance (between 2 and 5%) in all tissues (see Fig. 4a).

From these experiments, and from Northern blot experiments (not shown), we concluded that all tissues tested co-express both N and H type mRNAs, in a varying ratio, with H-type always prevailing.

## N and H type human aldolase A mRNAs are coexpressed in hepatomas

Aldolase A isoenzyme is known to be reexpressed in hepatoma cells where it replaces the adult liver-specific isoenzyme, aldolase B (8). We show in Fig. 5 that abundance of both N and H-type aldolase A mRNAs was notably increased in human hepatomas as compared to normal liver.

In the case of rats submitted to a carcinogenic diet, it has been shown that this re-expression is mainly due to actively proliferating cell types, oval and transitional liver cells, which infiltrate liver lobules (20, 21, 22).

## N and H type mRNAs are induced by growth promoting agents

We next examined the concentration of the different aldolase A mRNA species in two types of human cells stimulated to proliferate by serum or other growth-promoting agents.

In human blood lymphocytes, stimulation either by serum or by serum plus phytohemagglutinin (PHA) which induces cell proliferation in this system (16) resulted in an increase of the two smaller N-type transcripts, of about 15 fold at the 24th hour as quantified by densitometry scanning of RNAse H analysis (Fig. 6) and Northern blot analysis. We did not detect, in this system, any noteworthy accumulation of H-type transcripts. This induction of under-represented mRNAs (N-type) fits well with the small increase of aldolase A activity (2 fold) in these cells (23).

Stimulation by 10%(V/V) serum of human hepatoma Hep G2 cells grown in a serum-free medium, resulted in a 7-fold increase of both N and H-type mRNA level, between the 15th and the

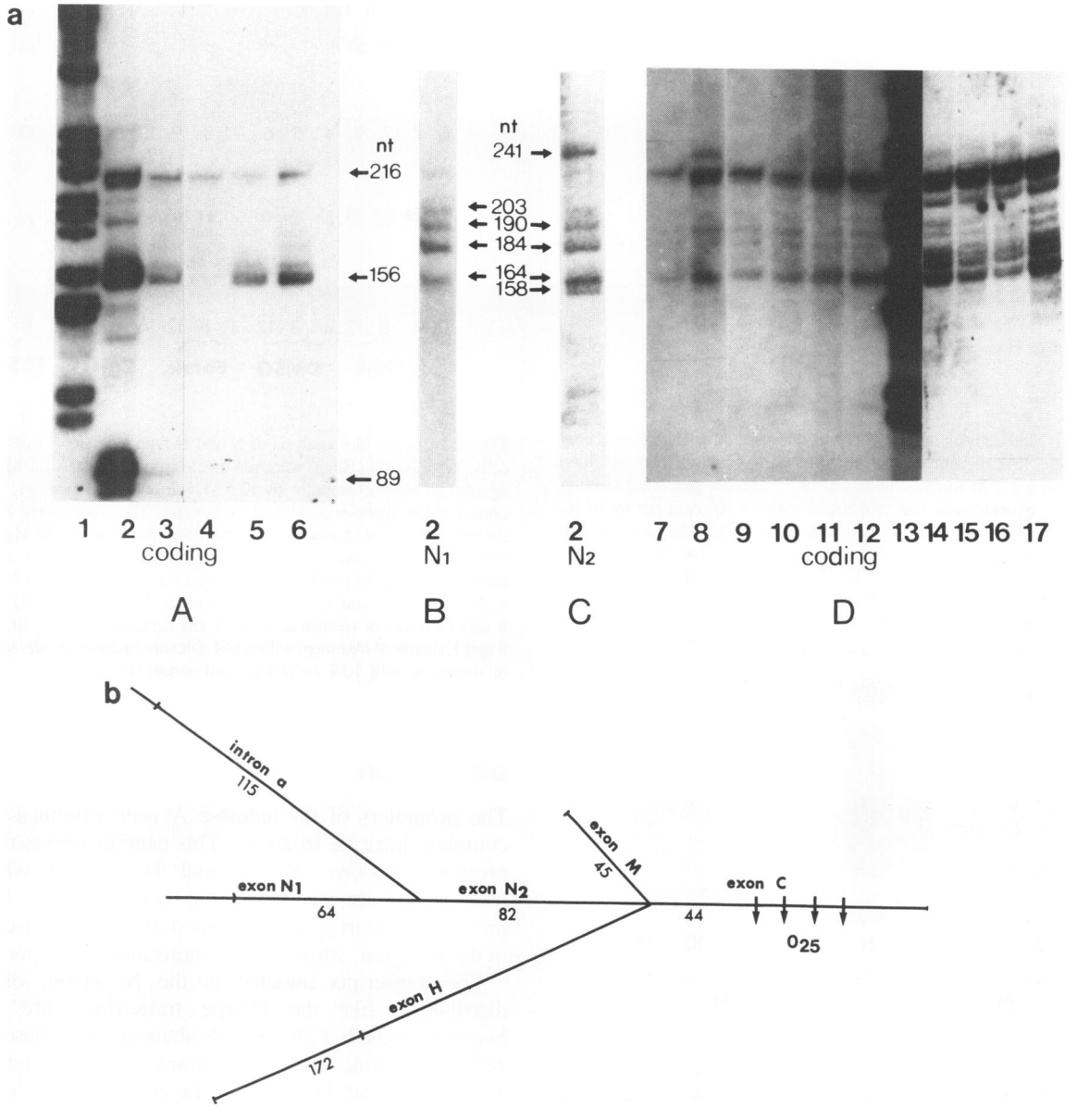


**Fig. 4:** RNAse H mapping analysis. **a:** Autoradiogram of RNAse H mapping. 20 $\mu$g of total RNAs were specifically digested by RNAse H, then denatured with 90% (v/v) formamide in 1×TBE buffer at 90°C and electrophorised in a denaturating 7 M urea, 5% (w/v) polyacrylamide gel. Migration products were blotted onto a nylon (gene screen-plus) membrane and hybridized with either a coding single-stranded probe complementary to the 5′ extremity of exon C1 (A and D), with a probe complementary to exon $N_1$ (B) or with a probe complementary to exon $N_2$ ( C). RNAs from: adult skeletal muscle (2), fetal skeletal muscle (3), blood lymphocytes (4), adult heart (5), fetal heart (6), fetal lung (7), adult lung (8), fetal spleen (9), adult spleen (10), fetal adrenals (11), adult adrenals (12), Hep G2 cells deprivated of serum for one day (14), two days (15), three days (16), and Hep G2 cells stimulated by serum for 20 hours (17). Lanes 1 and 13 show size standards. **b:** Scheme of the different 5′ ends of aldolase A mRNAs obtained after RNAse H specific digestion. Digestion by RNAse H of the mRNA/$O_{25}$ oligonucleotide duplex in exon C region (codons 8 to 16) is visualized by vertical arrows. The total length of the fragments generated by RNAse digestion, expressed in base pairs, can be calculated by addition of the numbers between each start site of transcription and oligonucleotide $O_{25}$.

24th hour of treatment (Fig. 4a−D, lanes 14 to 17, and Fig.7, lanes 0 and 13) RNAse H mapping analysis showed that it was mainly the two most downstream transcription initiation sites located in exon N1 and the most usptream and downstream sites in the H promoter region which responded to serum (Fig. 4a−D, lanes 14 to 17). Treatment with phorbol 12-myristate13-acetate (PMA), a tumor-promoting agent known to interact with protein kinase C (24, 25), also increased the level of both N and H-type aldolase A mRNA (Fig. 7, lanes 0, 1, 2 and 3), but quicker than with serum (as soon as the 4th hour instead of the 8th hour).

In contrast, calcium ionophore A23187, and forskolin (an activator of adenylate cyclase) seemed to be relatively inefficient in stimulating accumulation of aldolase A mRNAs (Fig.7, lanes 10, 11, 12 and 7, 8, 9; respectively) in this cellular system.

### H promoter region can function independantly in transfection experiments.

The next step in our work was then to search for DNA elements controlling transcription of each mRNA species.

To determine whether N and H promoters could act independantly, we performed transfection experiments using constructs in which either N or H 5′ flanking regions, 1500 and 420 bp-long, respectively could drive the expression of the bacterial chloramphenicol acetyl transferase gene CAT. These

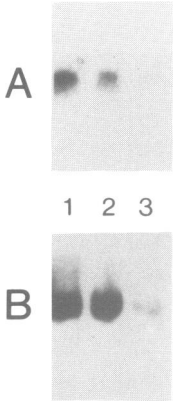


**Fig. 5:** Northern blot analysis of N and H-type aldolase A mRNA transcripts. Total RNAs were first denatured with 10 $\mu$M hydroxymethyl mercury, then electrophorized in a 2.2 M formaldehyde 1% (w/v) agarose gel. After blotting onto nylon filters, hybridization was performed with $2\times10^6$ cpm per ml of $N_2$ or H single stranded probes. Final washing was in 0.1 SSC, 1% (w/v) sodium dodecyl sulfate buffer at 65°C. Filters were exposed (for 3 days) at −80°C between two intensifying screens. **A:** $N_2$ probe. **B:** H probe. **A** and **B:** hepatoma n°1 (1), hepatoma n°2 (2), normal liver (3).

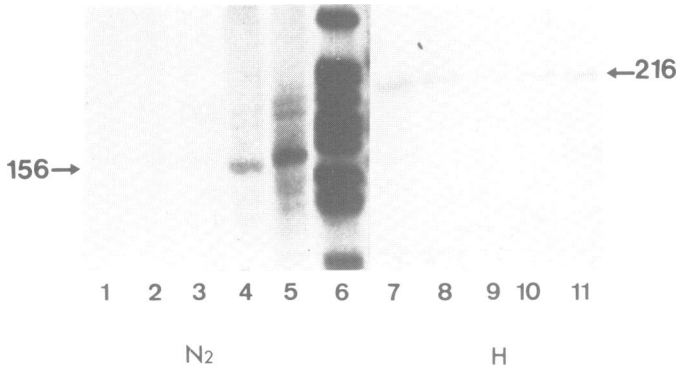


**Fig. 6:** Induction of the accumulation of aldolase A mRNAs in stimulated lymphocytes-**RNase H analysis.** mRNAs isolated from lymphocytes stimulated for 3 hours (lanes 1 and 11), 6 hours (lanes 2 and 10), 10 hours (lanes 3 and 9), 16 hours (lanes 4 and 8) or 48 hours (lanes 5 and 7) were digested with RNase H as described previously and revealed either with a probe encompassing exon N2 (lanes 1 to 5), or with a probe encompassing exon H (lanes 7 to 11). Size standards are shown in lane 6.

constructs called N-CAT and H-CAT( see Fig. 8b) were transfected in hepatoma Hep G2 cells (26), and in fibroblastic LTK$^-$ cells.

The results were drawn from at least two independant experiments using different plasmid preparations. The main findings of these experiments were the following:

a) Expression of the N-CAT constructs was always very low (about 0.1% of H-CAT). Fig. 8a−A and B shows the relative strength of N-CAT as compared to H-CAT and PSV2-CAT constructs in two cell types.

b) Expression of the H-CAT construct was, in contrast, very high in the two cell lines tested (Fig. 8a−A and B), with an activity similar to the one observed for the PSV2-CAT construct.

The 420 bp-long 5′ flanking region of exon H appears therefore to contain all the necessary DNA elements to constitute a very strong ubiquitous promoter.

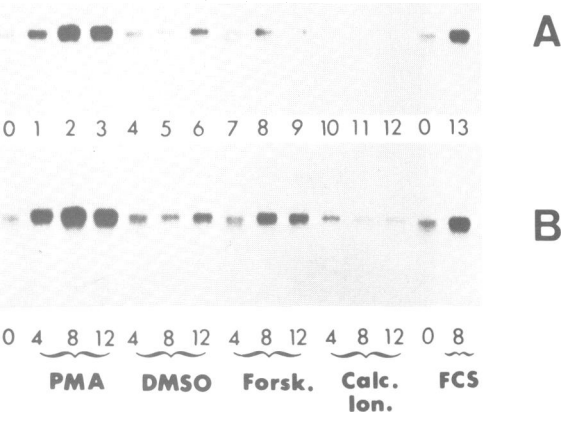


**Fig. 7:** Northern blot analysis of N and H type aldolase A mRNAs in Hep G2 cells. Total RNAs (10 $\mu$g) were run on electrophoresis in a 1.1 M formaldehyde-agarose gel after denaturation by 10 mM hydroxymethyl mercury, and then blotted onto a nylon (gene screen-plus) membrane. The filters were hybridized with $2\times10^6$ cpm per ml of single-stranded probes. **A:** $N_2$ probe. **B:** H probe. **O:** RNA from cells after 3 days of serum deprivation. **1, 2** and **3:** 4, 8 and 12 hours of treatment with 1 $\mu$M phorbol 12-myristate 13-acetate (PMA) in 0.1% (v/v) DMSO. **4, 5** and **6** : 4, 8 and 12 hours of treatment with 0.1% DMSO. **7, 8** and **9:** 4, 8 and 12 hours of treatment with 10 $\mu$M forskolin (forsk). **10, 11** and **12:** 4, 8 and 12 hours of treatment with 5 $\mu$M calcium ionophore (calc. ion.) **13:** 8 hours of treatment with 10% (v/v) fetal calf serum (FCS).

## DISCUSSION

The promoters of the aldolase A gene exhibit a surprisingly complex, intricate structure. This gene possesses three distinct promoter regions, N, M, and H, two of which initiate transcription in an extremely heterogeneous fashion. As previously reported, three major start sites of transcription exist in the H-region, whose relative utilization vary upon tissue type.

The transcripts initiated in the N region, of ubiquitous distribution like the H-type transcripts, are particularly heterogeneous, but 20 fold less abundant than these latter ones. At least 8 distinct start sites of transcription are detected within a 120 base pair DNA fragment encompassing 'exon N1' as previously defined (2), and the region just following it (intron *a*, between so-called N1 and N2 exons).

Such an heterogeneity of transcription initiation sites is a common phenomenon in housekeeping genes, found often associated with GC boxes (27, 28) and lack of consensus TATA boxes (29, 30). This is the case for the strong ubiquitous H promoter studied in this paper, but not for the N region itself, which behaves as a weak diffuse promoter. One hypothesis is that this region does not possess its own, cis-activating elements but shares those of the efficient downstream H promoter. The scarcity of canonical promoter elements in that region, and the influence of distal activating sequences could generate the complex pattern of transcription initiation sites observed.

Downstream start sites of transcription mapped within intron *a* appear to be restricted to cardiac and skeletal muscle tissues. Since the M promoter is totally inactive in heart, we can rule out the hypothesis that utilization of these sites specifically in heart and muscle results entirely from activation of the M transcriptional apparatus. Interactions with specific regulatory factors in heart and skeletal muscle as opposed to non-muscle tissues could explain that transcriptional start site patterns, for both N and H regions, differ in muscle and non-muscle tissues. Elucidation of these interactions is now underway.

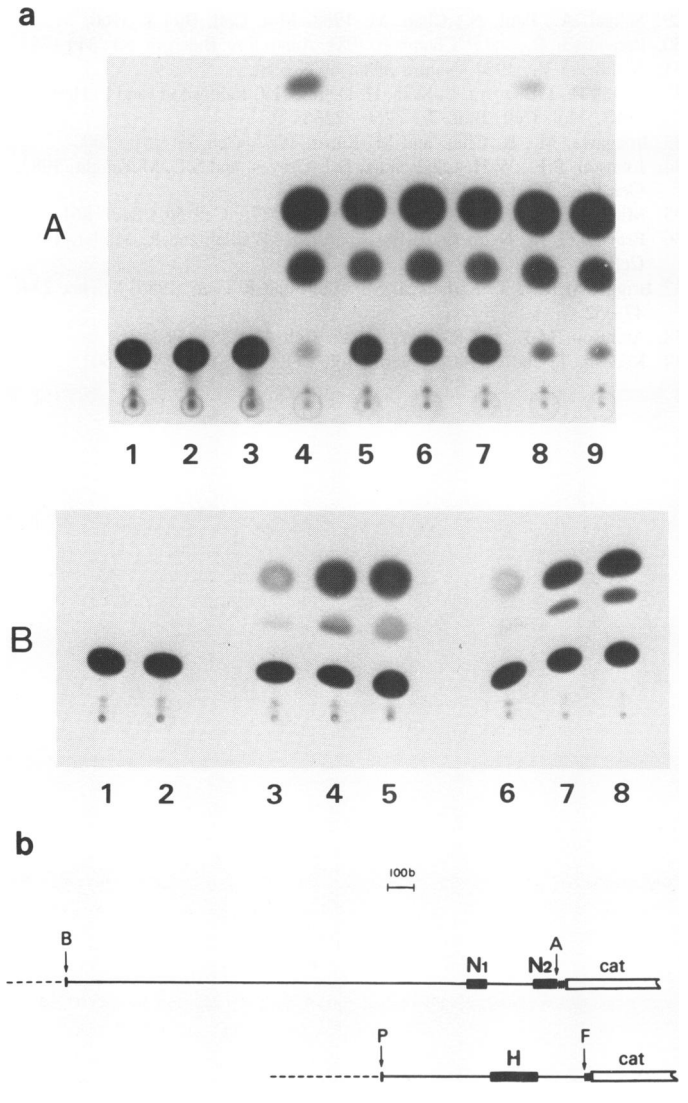


**Fig. 8:** Transfection experiments. **a:** Transient expression of N-CAT and H-CAT constructs into different cell types: chromatography analysis. **A**: Hep G2 cells were transfected with N-CAT(1,2 and 3), H-CAT (4, 5 and 6) and PSV2-CAT (7, 8 and 9) plasmids. CAT activity was measured with 40 $\mu$l of the homogenates in a one hour reaction, dilutions for essays 4 to 9 were performed (not shown). B: LTK$^-$ cells were transfected with N-CAT (1 and 2), H-CAT (3, 4 and 5) and PSV2-CAT(6, 7 and 8). CAT activity was measured in the homogenates with 40 $\mu$l in 1, 2, 4, 5, 7 and 8, or 5.$\mu$l in 3 and 6 in a one hour reaction. CAT activity was determined, and standardized by a luciferase essay. The ratios between the CAT activities of the different plasmids were not significantly different in both cell types and the ratio of H-CAT activity to N-CAT activity varied from 0.8 to 2.**b:** Schematic map of CAT constructs including CAT sequences and N or H exons and flanking regions. **B** = BamH1 (artificial site at position O as defined in previous paper (2)) upstream of exon $N_1$. **A** = Asp700 position 1850, in exon $N_2$. **P** = $Pst_1$ position, 2692 upstream from exon H. **F** = $Fsp_1$ position 3454, in intron following exon H of the human aldolase A sequence. $pEMBL_{19}$ polylinker sequences are figured as a hatched region.

Induction of the expression of mRNAs encoding enzymes of the glycolytic pathway is a well-known phenomenon in growing cells. We have shown that this is also the case for N and H type mRNAs, *in vivo* in hepatoma, and in cultured Hep G2 cells treated with serum. Phorbol ester PMA, which stimulates the protein kinase C pathway (for a review see Nishizuka, 31), can mimick this effect, although it acts more rapidly than serum.

The fact that the induction we observed is, at least in part, regulated at the transcriptional level is suggested by S1 nuclease mapping experiment using probes encompassing both intronic and exonic regions, that show a parallel increase in precursor and mature mRNA species in Hep G2 cells, and by transfection experiments that reproduce this inductibility (S. Gautron and P. Maire, unpublished results). Investigation of the 420 bp upstream of aldolase A H-type promoter has led us to notice several potential binding sites for known 'phorbol ester response' regulatory proteins: two $AP_1$ consensus binding sites (32) (TGAGTCA at positions 2835 and 2917, which are 100% conserved with the rat aldolase A gene (4), at least one $AP_2$-like binding sites (33) (CGGGCG approximately at 2721) and one SV40 'core enhancer' putative binding site for C/EBP, AP3 (34, 35) (TGTGGT at position 2908). The hypothesis that these sites could account for serum and PMA response will be investigated.

The response of human lymphocytes to serum differ drastically from that exhibited by Hep G2 cells: only the two smallest N-type transcripts accumulate up to 15 fold during treatment by serum while H-type mRNA level does not vary sgnificantly.Such an heterogeneity of response depending on cell line has already been reported for c-fos expression, whose mRNA concentration is increased by cAMP in macrophages but not in fibroblasts (36). In the case of the Aldolase A gene, we can conclude partially different mecanisms can lead to either N or H-type mRNA accumulation.

A first question arising from this series of results was whether the three promoters of the aldolase A gene could act independantly of one another and if the N and H promoters were each flanked with DNA elements responsible for their ubiquitous expression, and we chose to study the 5′ flanking regions of each promoter by transfection of CAT constructs in cultured cells.

An H-CAT construct, containing 420 bp 5′ to the usptream initiation site of exon H proved to be particularly efficient in the cell lines tested. An explanation for this strength could reside in the numerous and clustered characteristic DNA motifs that figure upstream from the H promoter and which are known to bind regulatory proteins, such as 8 GC motifs, known to interact with the ubiquitous protein SP1 (37).

In order to search for regulatory DNA elements, a N-CAT plasmid was tested containing the N-region from 1.5 kb 5′ to 'exon N1' region to part of exon N2. This construct was very weakly expressed after transfection in Hep G2 and LTK$^-$ cells.

We thus propose that at least some indispensable regulatory regions of DNA lie elsewhere, for instance in the H-type regulatory region; in favour of this possibility is the fact that expression of N and H type mRNAs is completly coordinated, in time and intensity in Hep G2 cells. Such cases where a unique DNA regulatory element can control two distinct promoters in artificial constructs have been already reported for the $\varkappa$ light chain enhancer (38) and for the SV40 enhancer (39), and this possibility greatly deserves to be tested.

In conclusion, we have described the intricate structure of the multiple promoters of the human aldolase A gene. Two of these promoters generate extremely heterogeneous transcripts that differ in their 5′ end ; they both function ubiquitously in human tissues, and can be induced in several conditions of cell proliferation: e.g., *in vivo* in hepatoma tissue, and, in cultured cells after stimulation by serum and tumour-promoting agents. Promoter H encompassed within a 420 bp fragment 5′ to the upstream start site of transcription of exon H functions autonomously at a very high level in different transiently transfected cell lines. In contrast, a construct in which the reporter gene is driven by the N region functions very weakly in transfected cells. Further investigations

using cultured cells and transgenic mice will be needed to precise the functional interelations between the three promoter regions of the aldolase A gene.

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