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# Murine mRNA for the β-subunit of integrin is increased in BALB/c-3T3 cells entering the $G_1$ phase from the $G_0$ state

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The amount of murine mRNA for the β-subunit of integrin is enriched 6-fold when BALB/c-3T3 cells traverse from the $G_0$ state to the $G_1$ phase, whereas it remains at the basal level when the cells are growing continuously. The peak of its appearance is at 10 h after serum stimulation. The increase in integrin mRNA at a specific point in cell proliferation may be correlated with growth-signal transduction.

Cell cycle; Cell growth; $G_0/G_1$ transition; Integrin; Fibronectin receptor

## 1. INTRODUCTION

In cells entering the cell cycle from the resting state, several genes are specifically activated by growth factors. In particular, the early responses in this process have been investigated intensively [1–6]. On the other hand, there are few reports about late appearing products that are specific for the $G_0/G_1$ transition [7,8]. Therefore, I started to study genes which are activated at 10 h after serum stimulation of resting BALB/c-3T3 cells [8]. Here, I report that the amount of murine mRNA for the β-subunit of integrin is increased only when BALB/c-3T3 cells traverse from the $G_0$ state to the $G_1$ phase, whereas it remains at the basal level when the cells are growing continuously.

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*Abbreviations:* dT, deoxythymidylic acid; poly(A), polyadenylic acid; dCTP, deoxycytidine 5′-triphosphate

The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession no. Y00769

## 2. MATERIALS AND METHODS

BALB/c-3T3 cells (clone A31) were provided by Dr C. Stiles (Harvard Medical School). Oligo(dT)-cellulose was from Sigma. A cDNA synthesis system and a multiprime DNA labeling system were purchased from Amersham. λgt10 phage and Bluescript plasmid were from Stratagene Cloning System. Exonuclease III and mung bean nuclease were from Takara Shuzo. [$\alpha$-$^{32}$P]dCTP (spec. act. ~3000 Ci/mmol) was from New England Nuclear.

Total cytoplasmic RNAs were extracted [9,10] from BALB/c-3T3 cells which had been stimulated by serum addition for 10 h as described [8]. Poly(A) RNAs were selected by the oligo(dT)-cellulose column procedure, and a cDNA library was constructed [11] using λgt10 phage. Differential hybridization screening was carried out as in [4]. cDNA probes for screening were synthesized by reverse transcriptase (Seikagaku Kogyo) in the presence of [$\alpha$-$^{32}$P]dCTP, using poly(A) RNAs from either quiescent or serum-stimulated cells. Northern blotting was performed using glyoxal and dimethyl sulfoxide [12]. Probes for Northern hybridization were labeled with [$\alpha$-$^{32}$P]dCTP by the multiprime labeling method [13]. Hybridization was carried out at 42°C and the filter (Zeta probe, Bio Rad) was washed at 50°C in 0.1 × SSC, 0.1% SDS solution [12]. Densitometry of the autoradiogram was carried out using an Elscript 400 densitometer (Hirschmann). For nucleotide sequence analysis, cDNA was subcloned into Bluescript plasmid. Using exonuclease III and mung bean nuclease, plasmids containing inserts of various lengths were obtained [14]; thereafter the nucleotide sequence was determined by the chain-termination DNA-sequencing method [15,16].

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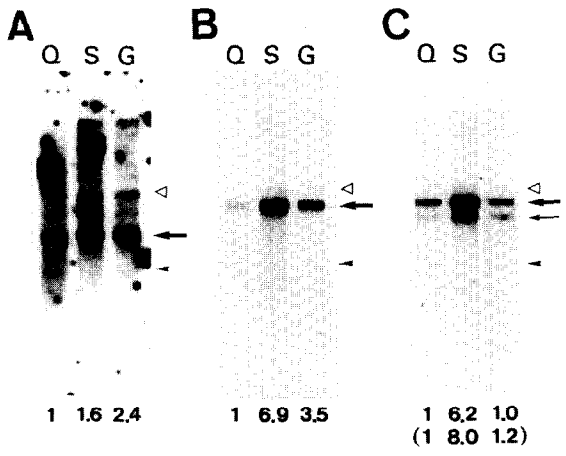


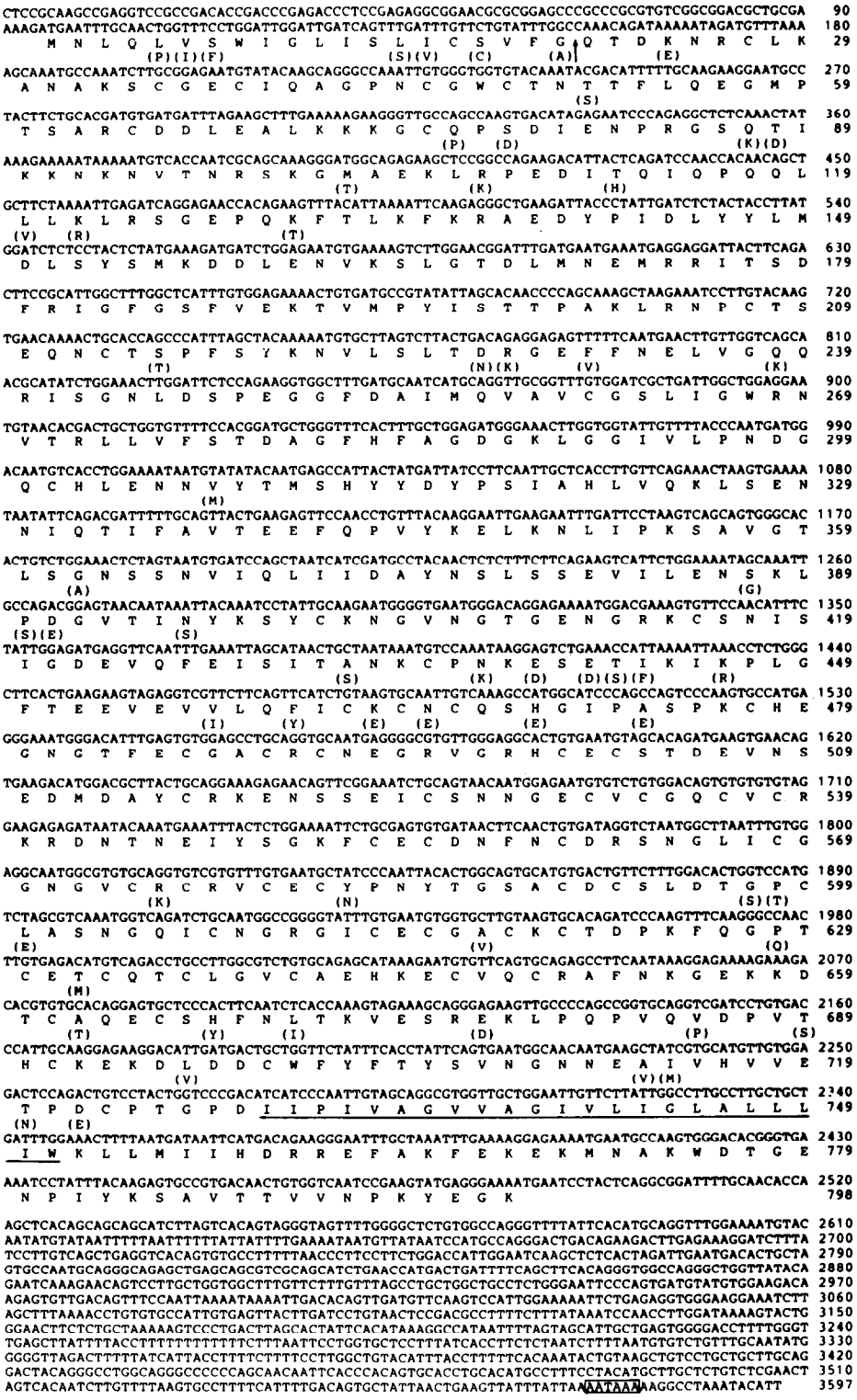

Fig.1. Increased mRNAs in growth-stimulated BALB/c-3T3 cells. Total cellular RNAs were extracted from quiescent cells (Q), cells at 10 h after serum stimulation (S), or continuously growing cells that had reached 70% confluency (G) [8]. RNAs were further processed using an oligo(dT)-cellulose column to obtain the poly(A) RNAs. The poly(A) RNAs (0.5 $\mu$g) were loaded on a 1.1% agarose gel for Northern blotting. cDNA fragments were excised from λgt10 phages numbered clone 83 (A), clone 49 (B), and clone 55 (C), and labeled with [$\alpha$-$^{32}$P]dCTP. Hybridization was carried out as described in section 2. The open triangle and arrowhead correspond to the positions of 28 S and 18 S ribosomal RNA, respectively. The arrows indicate the positions of hybridized mRNAs. The values at the bottom show the variation in intensity measured by densitometry. Values in parentheses in C are for the lower band shown by the thinner arrow. The intensity of Q was taken as unity in each case.

## 3. RESULTS AND DISCUSSION

Out of $6.5 \times 10^5$ plaques, 209 showed stronger signals in plaque hybridization with cDNAs from stimulated cells than those from resting cells. Different intensities were detected in 22 out of 209 clones by repeated differential screening. Next, these clones were examined by Northern hybridization using poly(A) RNAs from resting, growth-stimulated, or continuously growing cells. Densitometry indicated that in 7 out of 22 clones, the intensities of growth-stimulated samples were over 1.5-fold those of the resting ones. These clones could be classified into three types as shown in fig.1. The first type (2 clones) showed that the level of mRNA was increased during the $G_0/G_1$ transition, but underwent a greater increase in the continuously growing cells (fig.1A). In the second group (4 clones), the increase in mRNA was greater in the $G_0/G_1$ transition than in the growing state (fig.1B). In the third category (1 clone), the proportion of mRNA in total poly(A) RNA remained at the basal level in both the $G_0$ and continuously growing states, and increased only in the $G_0/G_1$ transitional phase (fig.1C).

The third category was intriguing and therefore the nucleotide sequence of clone 55, consisting of 289 nucleotides, was determined as described in section 2. Except for the 15 deoxyadenylate residues located at the 3′-end, there was one open reading frame composed of 90 amino acids. This nucleotide sequence was later found to correspond to nucleotides 87–375 of ST1 cDNA (fig.2). It is conceivable that the A-rich region from nucleotide 361 to 375 served as an annealing site for the oligo(dT) primer during the first strand synthesis in the course of cDNA library construction. The homology search in the GenBank Library revealed that this deduced amino acid sequence showed 67.8% homology with the N-terminal region of chicken integrin [17]. A clone possessing a nearly full-length cDNA insert was then selected from the original cDNA library. Out of $1.5 \times 10^5$ plaques, 47 clones gave positive signals with the labeled fragment from clone 55. Clone ST1, which contains a 3.7 kb insert, was finally obtained. The entire nucleotide sequence was determined as described in section 2 (see fig.2). This cDNA exhibits very high homology with the $\beta$-subunit of the human fibronectin receptor [18]. The homology was 87.6% in the nucleotide sequence and 92.2% in the amino acid sequence. The deduced amino acid sequences were identical except for the amino acids shown in parentheses in fig.2. The small difference in the amino acid sequences may be explained by a species difference. It is also notable that the 76 amino acids in the C-terminal region, which includes the putative cytoplasmic

Fig.2. cDNA sequence and deduced complete amino acid sequence of ST1. The nucleotide sequence of ST1 cDNA was determined as described in section 2. The putative signal peptide cleavage site is designated by an arrow [24]. The putative transmembrane domain is underlined [17,18]. The poly(A) signal is boxed. Amino acids in parentheses correspond to deduced amino acids in the human fibronectin receptor $\beta$-subunit [18].



and transmembrane domains (shown in fig.2), were completely identical. Therefore, ST1 is the murine cDNA of the $\beta$-subunit of the fibronectin receptor, which has also been designated integrin [17,19].

To confirm that ST1 mRNAs are increased when quiescent BALB/c-3T3 cells enter the $G_1$ phase but not increased when cells are growing continuously, the time course of the appearance of this mRNA was studied (fig.3 and table 1). The peak in the distribution of this mRNA among the total cellular RNAs was at 10 h after serum addition, just before DNA synthesis [20,21]. In the case of growing BALB/c-3T3 cells at 30% (fig.3, lane 9) and 70% (fig.3, lane 10) confluency, the intensities of the signal were of the same level as that in the case of quiescent cells. Total cellular RNAs from cells at 30, 40 and 50 h after serum stimulation were also studied in the same experiment, but the intensities of the bands were below the level of detectability (not shown). Thus, it is conceivable that ST1 expression is increased at a certain point in the $G_0/G_1$ transition and not increased when the cells are growing continuously. It is also interesting that ST1 mRNAs consist of 2 species of about 3.8 and 3.2 kb (figs 1,3), and the variations in intensities were similar (table 1). From the nucleotide numbers, ST1 cDNA, shown in fig.2, corresponds to the upper band in these Northern blots. The appearance of the shorter mRNA may be a consequence of alternative splicing or be due to the presence of a very similar gene.

It has been reported that integrin connects extracellular matrix proteins, such as fibronectin, and intracellular cytoskeletal components [19]. Since integrin is fundamental for cell attachment, there must be continuous expression of this protein at a considerable level, especially in monolayer cultures like BALB/c-3T3 cells. Thus, even a 6-fold increase could be judged as considerable activation. Matsuhisa and Mori [22] reported that anchoring is necessary only at a certain point in the $G_1$ phase in order for cells to proceed to DNA synthesis. Bockus and Stiles [23] reported alterations in the organization of microfilaments and microtubules during the $G_0/G_1$ transition. Cell anchoring or a change in cell shape may be required at a particular point during the $G_0/G_1$ transition. In this case, the activation of the integrin gene is necessary at or before that time. Various cell sur-

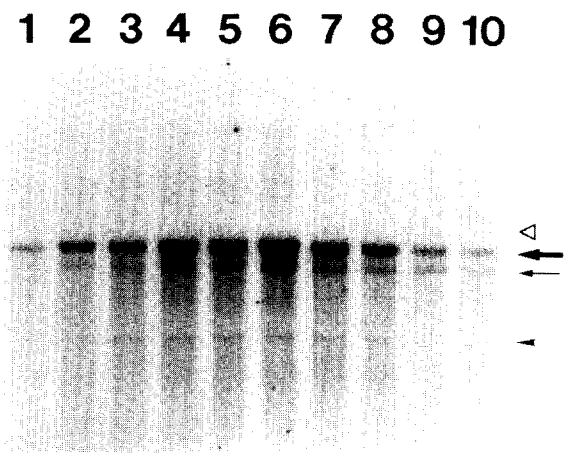



Fig.3. Time course of appearance of ST1 mRNA in serum-stimulated BALB/c-3T3 cells traversing from the $G_0$ state to the $G_1$ phase. Total cytoplasmic RNAs were extracted from quiescent cells (lane 1) or cells at 2 (lane 2), 4 (lane 3), 6 (lane 4), 8 (lane 5), 10 (lane 6), 15 (lane 7) and 20 h (lane 8) after serum addition. RNAs were also extracted from growing cells that had reached 30% (lane 9) and 70% (lane 10) confluency. 5-$\mu$g samples of total cellular RNAs were loaded on an agarose gel and processed for Northern hybridization using the labeled cDNA fragment from clone 55 as in fig.1. The thick and thin arrows indicate the positions of large and small ST1 mRNAs, respectively. The open triangle and the arrowhead denote the positions of 28 S and 18 S ribosomal RNAs, respectively.

Table 1

Variations in intensity of ST1 mRNAs with time elapsed after addition of serum to quiescent cells

| | Upper band | Lower band |
|---|---|---|
| Q | 1 | 1 |
| 2 h | 1.92 ± 0.21 | 1.87 ± 0.07 |
| 4 h | 2.71 ± 0.33 | 2.57 ± 0.43 |
| 6 h | 3.80 ± 0.94 | 3.26 ± 0.48 |
| 8 h | 4.29 ± 1.80 | 3.53 ± 0.75 |
| 10 h | 6.21 ± 2.90 | 4.08 ± 0.76 |
| 15 h | 3.99 ± 2.95 | 2.98 ± 0.61 |
| 20 h | 2.09 ± 1.41 | 2.06 ± 0.28 |
| G-1 | 1.03 ± 0.55 | 1.55 ± 0.41 |
| G-2 | 0.78 ± 0.49 | 1.00 ± 0.21 |

Three experiments were performed as in fig.3, and Northern blots were processed for densitometry. The intensities with time elapsed from 2 to 20 h after serum addition were measured. Intensities of quiescent (Q) and continuously growing cells that had reached 30% (G-1) or 70% (G-2) confluency were also measured. The value at quiescence was taken as unity in each case. Values of the mean ± SD were calculated and are shown

face molecules share the common $\beta$-subunit of integrin, coupling with different $\alpha$-subunits [19]. Therefore, the increase in mRNA for the $\beta$-subunit of integrin can be the result of total synchronous activation of the integrin family or a consequence of the requirement of some specific molecule, such as the fibronectin receptor.

Investigation of the level of expression of the protein product of the $\beta$-subunit and studying of the variation in $\alpha$-subunits will be necessary to gain understanding of the functions of integrin in cell proliferation.

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