GENETIC CONTROL OF ALPHA-FETOPROTEIN SYNTHESIS IN THE MOUSE*

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Alpha-fetoprotein (AFP)[1] is one of the major plasma proteins in mammalian fetuses, but it disappears almost completely after birth (1–3). In man the maximal level in the fetus is about 3 mg/ml, which decreases to a basal level of approximately 2–20 ng/ml in the adult (see reference 3). The concentration therefore declines more than 10^5-fold during development, and a very efficient control mechanism must operate to switch off the synthesis of AFP after birth. A similar drastic decrease in AFP concentration after birth occurs in mice. However, the basal level of AFP in the serum of adult mice is somewhat higher than in man (4). High serum AFP concentrations are also found in connection with primary liver cancer and teratocarcinoma (2, 5), and measurement of serum AFP level is widely used for the diagnosis of these tumors.

The properties and occurrence of AFP suggest that this protein plays an important role both in normal embryogenesis and in various diseases. The chemistry and distribution of AFP have therefore been studied in detail (see references 2, 3). In contrast, virtually nothing is known about the genetic control of this protein. Knowledge about the genetics of AFP could contribute to the understanding of the process of malignant transformation and also give information of general importance concerning the genetic control of embryonal proteins. In particular, it should be of interest to gain knowledge about the mechanism that switches off AFP synthesis after birth. To approach the genetics of this mechanism, we assumed that a change in this mechanism might lead to a changed basal AFP concentration in adult mice. A mouse strain having an exceptionally efficient switch-off mechanism would be expected to have an unusually low basal AFP level, while a less efficient switch-off mechanism should lead to an unusually high basal level of AFP. We describe here a gene causing a remarkably high AFP concentration in adult mice.

Materials and Methods

Mice. All strains of mice except two were originally obtained from The Jackson Laboratory, Bar Harbor, Maine. BALB/c/BOM was obtained from Bomholtgaard Animals Ltd., Ry, Denmark, and the *Mus musculus castaneous* mice were kindly given to us by Dr. Eva Eicher, The Jackson Laboratory. Breedings were performed under standard conditions.

* Supported by grant B76-16X-04510-02A from the Swedish Medical Research Council, and grant CA 19894-01 from the National Cancer Institute.

[1] *Abbreviations used in this paper:* AFP, alpha-fetoprotein; RIA, radioimmunoassay.

Purification of AFP. AFP was purified from newborn mice according to Pihko and Ruoslahti (4). This procedure involves absorption of a crude lysate with an immunoadsorbent specific for mouse AFP. After elution from the immunoadsorbent with 8 M urea, contaminating proteins are removed with appropriate immunoadsorbents, and the preparation is finally fractionated on a Sephadex G-200 column. AFP produced in this fashion is free from contaminants, as measured in polyacrylamide gel electrophoresis and immunodiffusion tests (4).

Radioimmunoassay (RIA). Mouse AFP was assayed essentially as previously described (4). Purified mouse AFP was iodinated using the chloramine-T method (6). The standards and the mouse sera diluted more than 1:25 were tested in phosphate-buffered saline containing normal human serum diluted 1:25 as protein diluent. For the first step in the RIA, sheep anti-mouse AFP was used, and antibody-bound AFP was then precipitated using rabbit anti-sheep IgG serum. Normal sheep serum was added to give a final concentration of 1:2,000 to provide enough carrier γ-globulin for the precipitation reaction.

Antisera. Anti-mouse AFP was produced in a sheep by subcutaneous injections of purified mouse AFP supplemented with Freund's complete adjuvant. The injections, 0.5 mg each, were given every 2 wk. Antiserum obtained after 3 mo of immunization was used for the radioimmunoassay (RIA). Although no contaminating antibodies could be demonstrated in this antiserum by immunodiffusion tests, it was absorbed with normal mouse serum coupled to Sepharose (7). Anti-sheep IgG serum was produced in rabbits by immunizing with sheep IgG purified by DEAE cellulose chromatography.

Crossed Immunoelectrophoresis. This was performed according to Laurell (8) using 0.6% agarose and 0.075 M barbital buffer, pH 8.6. The gel used for the second step contained 0.5% rabbit anti-mouse AFP serum.

Results

Serum AFP Concentration in Adult Mice from Various Inbred Strains. The AFP concentration in serum from adult mice has been measured for 27 different inbred strains (Table I). The most striking result was the high level of AFP in BALB/c/J mice, where the average AFP concentration was 994 ng/ml. All other strains of mice, including two other substrains of BALB/c mice, had average AFP concentrations that were between 34 and 173 ng/ml. Also among these latter strains there appeared to be differences in AFP concentration. The AFP levels in A mice, for example, were considerably higher than in DBA/1 mice. These differences were less striking than the difference between BALB/c/J and other strains, and they will not be considered further in this paper. No difference in AFP concentration between male and female mice of the same strain was found in the three strains where this was tested.

Although the average AFP concentration in BALB/c/J mice was high, there was great variation between individual mice of this strain. Fig. 1*a* shows the distribution of AFP concentration for 30 individual BALB/c/J mice and for 31 individual mice of a strain showing a low AFP concentration, DBA/2. Whereas the AFP concentration in BALB/c/J varied between 250 and 2,330 ng/ml, all DBA/2 mice had concentrations below 100 ng/ml. The variation among BALB/c/J mice seems to be due to physiological variation and not to genetic differences, since repeated bleedings from individual mice of this strain showed similar variation (data not shown).[2]

Genetic Analysis. To study whether the high level of AFP in BALB/c/J mice

[2] The high and variable AFP level in BALB/c/J mice has been observed in mice obtained from The Jackson Laboratory at several different occasions as well as in BALB/c/J mice bred in our laboratory in Lund. Thus, the high AFP level appears to be a stable and characteristic property of this strain of mice.

TABLE I
Serum AFP Levels in Adult Mice from Various Inbred Strains

Strain	Age at bleeding	Number tested	Average AFP concentration*	Range
	wk		*ng/ml*	*ng/ml*
Female mice				
A	12–13	6	173	145–200
AKR	10	6	121	83–176
AU/Ss	8	6	82	68–106
BALB/c/BOM	8	12	63	37–105
BALB/c/By	8–14	13	46	24–79
BALB/c/J	9–10	30	994	250–2,330
B10.D2	11–12	3	91	87–94
BUB/Bn	11	4	103	84–135
CBA	10–14	6	42	23–46
C57BL/6	11	6	131	55–165
C57BL/10Sn	7–10	3	159	155–163
C57BL/Ks	13	4	52	44–66
C57L	14	4	49	42–65
C3H/He	8	6	133	105–160
DBA/1	8–14	6	34	20–37
DBA/2	12	31	47	20–83
I/Ln	8–14	6	118	88–149
LP	9	6	99	74–117
M.m. castaneous	30	2	51	51‡
NZB	8	6	60	51–90
PL	14	4	90	51–115
RF	13	4	77	71–84
RIII/2	11	4	38	33–45
SEA/Gn	10	4	63	47–74
SJL	9	6	65	61–77
SM	9	4	129	86–160
129/Sv	8	6	115	73–156
Male mice				
BALB/c/By	8–14	14	40	29–92
BALB/c/J	12	6	915	420–1,330
C3H/He	8	5	111	88–128
F_1 mice				
(BALB/c/J × DBA/2)	12–15	12	53	23–86

* AFP concentration was measured by RIA as described in Materials and Methods.
‡ Both mice had the same APF level.

is due to a genetic factor, this strain was crossed with one of the strains having a low AFP level, DBA/2. The data in Table I show that the F_1 mice from this cross had a low serum level of AFP, indicating that the high level of AFP in BALB/c/J mice is a recessive property.

A back-cross analysis was then performed (Fig. 1*b*). BALB/c/J mice were crossed with F_1 (BALB/c/J × DBA/2) mice, and 75 progeny mice were tested. Among these mice 38 had AFP levels between 170 and 1,480 ng/ml, and 37 had values between 33 and 99 ng/ml. This result is in perfect agreement with the 1:1 ratio expected for a Mendelian gene.

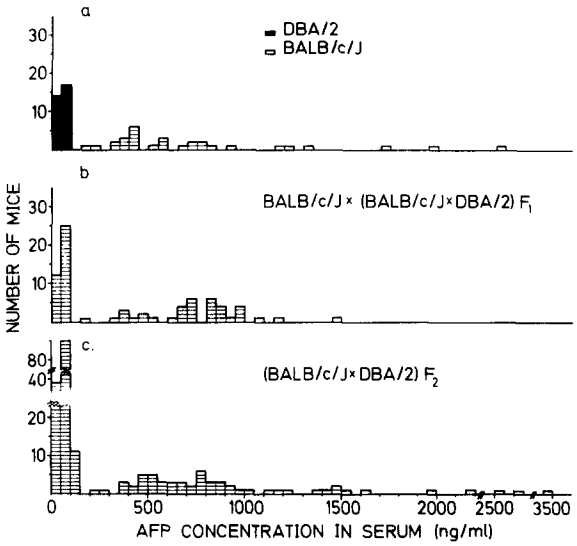

FIG. 1. Genetic analysis of the high serum AFP concentration in BALB/c/J mice. All mice were bled at an age of 9–12 wk.

The F_2 progeny of the cross can be separated into two classes according to their serum level of AFP (Fig. 1*c*). One of these classes shows low AFP levels like the DBA/2 parent, and the other class shows high AFP levels, with a broad distribution similar to that of the BALB/c/J parental strain. Assuming that all F_2 mice that have an AFP level below 150 ng/ml belong to one population and that all those with higher values belong to another population, one arrives at a ratio between the two populations of 2.1:1 (124:58). This is not in very good agreement with the 3:1 ratio expected for a Mendelian gene, and a chi-square analysis of this result shows that the difference from 3:1 ratio is weakly significant ($0.025 < P < 0.05$). However, in view of the result of the back-cross, it seems likely that the high AFP level in BALB/c/J mice is controlled by a single Mendelian gene. Since this gene affects the regulation of AFP synthesis, it has tentatively been given the name *Raf* (for regulation of alpha-fetoprotein). Attempts to locate this gene on the genetic map of the mouse have not been successful so far, but have shown that it is not linked to the T locus or to any of the coat color genes *a*, *c*, or *d*.

AFP Levels in Serum During the First Postnatal Months. The experiments described above have shown that adult BALB/c/J mice have considerably higher AFP levels than all other strains tested. Since the serum concentration of AFP drops very drastically during the first weeks of life, it was of interest to compare BALB/c/J mice and other mice in this respect.

Fig. 2 shows how the AFP concentration decreases with time in BALB/c/J mice and in two control strains, C3H/He and BALB/c/BOM. For all three strains the AFP concentrations varied considerably during the 1st wk of life. The data suggest, however, that all three strains have a similar AFP concentration in serum at birth. The AFP concentration starts to decrease at about 1 wk of age, but this decrease occurs faster in the control mice than in the BALB/c/J mice. According to the data in Fig. 2, AFP disappears from serum with a half-life of

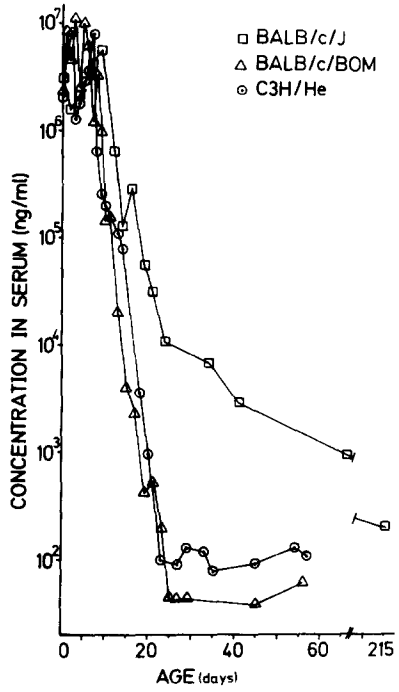

FIG. 2. AFP concentration in serum during the first postnatal months. For day 0–14, serum from several mice of the same litter was pooled, since only very small amounts of serum can be obtained from mice of this age. From day 15 and on individual mice of BALB/c/BOM and C3H/He were bled. For strain BALB/c/J several mice were bled also after day 15, and the average concentration of these sera was determined. The reason for this is that there is great variation in AFP concentration among individual BALB/c/J mice of the same age, as shown in Fig. 1*a*.

about 24 h in C3H/He mice and BALB/c/BOM mice. In BALB/c/J mice the initial rate of disappearance appears to be similar to that in the controls, but the rate then decreases, and the AFP concentration appears to decrease slowly during many months.

Biological Half-Life of AFP in BALB/c/J Mice. There could be two reasons for the high serum level of AFP in BALB/c/J mice: first, it could be the result of an increased synthesis of AFP; second, it could be the result of a decreased catabolism of AFP in these mice. To study this question, we have followed the elimination of ^{125}I-labeled AFP from the serum in BALB/c/J mice and in a control strain, BALB/c/By. The results show that there is a similar catabolic rate of AFP in the two strains (Fig. 3). Furthermore, the data show that the half-life of AFP in adult mice is about 24 h, i.e., it is the same as when AFP is rapidly eliminated from serum shortly after birth (Fig. 2). These results indicate that AFP is not catabolized with a decreased rate in BALB/c/J mice.[3]

[3] It could be argued that the rate of catabolism is slow in young BALB/c/J mice, as compared to other strains, and that it eventually increases, to become similar in adult mice of the various strains. This would lead to a slower disappearance of AFP in young BALB/c/J mice, as seen in Fig. 2. However, the shape of the curve in Fig. 2 shows that the rate of disappearance decreases with age in BALB/c/J mice.

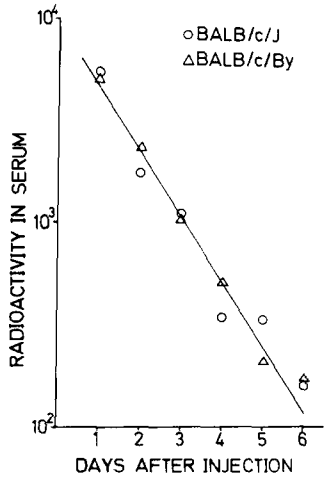

FIG. 3. Biological half-life of AFP in BALB/c/J mice and a control strain BALB/c/By. For each of the two strains, three mice were injected intravenously in the tail with 200 μl of ^{125}I-labeled mouse AFP in PBS. The dose of AFP injected corresponded to 9.8×10^4 counts per 100 s. One of the three mice was bled on days 1 and 4, one on days 2 and 5, and one on days 3 and 6, and the radioactivity in the serum was determined (counts per 100 s in 200 μl serum).

Histology. Inasmuch as various diseases of the liver as well as liver regeneration are associated with increased serum AFP levels (2, 3, 9), it was of interest to make a histological examination of livers from BALB/c/J mice. No sign of disease or other abnormality was found.

Direct Immunochemical Demonstration of Increased AFP Level in BALB/c/J Serum. In the experiments described so far, AFP concentrations were always determined by RIA technique (i.e., a technique where the presence of AFP in the test sample causes inhibition of binding of ^{125}I-labeled AFP to specific antibodies). Due to the high specificity of RIA methods, it is very likely that inhibition of binding in our system can be caused only by AFP, and that the RIA method is a safe method for the determination of AFP concentration. Nevertheless, we have confirmed that BALB/c/J mice have an increased serum level of AFP using a direct immunochemical method, the two-dimensional crossed immunoelectrophoresis technique of Laurell (8). The results (Fig. 4) show that BALB/c/J serum indeed contains increased levels of AFP. This AFP migrates in the same manner as purified control AFP, and the quantitation performed by this technique gives a value similar to that obtained by the RIA method.

Discussion

The experiments described in this paper were based on the assumption that a change in the mechanism that normally turns off AFP synthesis might affect the basal level of AFP in adult mice. The basal level of AFP was therefore determined for 27 different inbred strains of mice, and it was found to be considerably increased in one of these strains, BALB/c/J. In this strain the average AFP level is about 10-fold higher than in other strains at 9–10 wk of age. Although the average AFP level was much higher in BALB/c/J mice than in

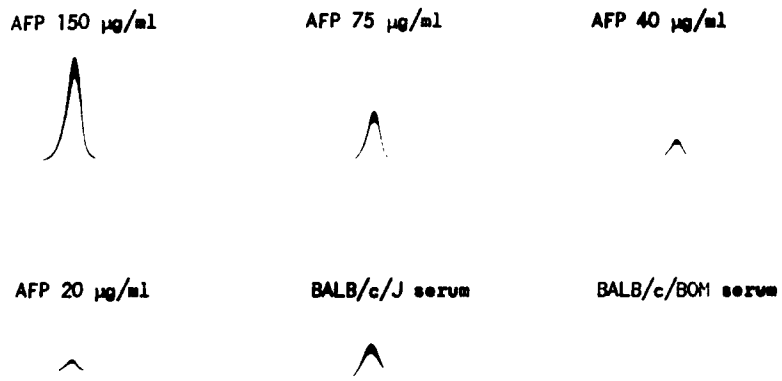

FIG. 4. Crossed immunoelectrophoresis (8) of serum from BALB/c/J and BALB/c/BOM mice, and of control preparations containing pure AFP. Since the serum concentration of AFP in adult mice is too low to be detected by this technique, the serum samples analyzed here were taken from mice 21 days of age, at which age the serum AFP concentration has not yet dropped to the low levels seen in adult mice (see Fig. 2).

other strains of mice, there was great variation among the BALB/c/J mice, causing some of them to have AFP levels not much higher than those found in other strains. However, in no case was the AFP level in BALB/c/J mice as low as in any of the other strains. This was important for the genetic analysis, which was performed by crossing BALB/c/J mice with one of the other strains, DBA/2. The F_1 mice from this cross had as low AFP levels as the DBA/2 parental strain, showing that the increased AFP level in BALB/c/J mice is a completely recessive property. The results of the back-cross $F_1 \times$ BALB/c/J strongly suggest that the increased AFP level in BALB/c/J mice is controlled by a single recessive Mendelian gene. The analysis of F_2 mice from the same cross showed less perfect agreement with the result expected for a Mendelian gene. However, the difference between the ratio observed and the expected 3:1 ratio is only weakly significant, and when all data are taken together, it seems very likely that the increased AFP level of BALB/c/J mice is controlled by a single Mendelian gene. We have tentatively named this gene *Raf* (for regulation of alpha-fetoprotein).

The kinetic studies, shown in Fig. 2, demonstrate that the rate with which AFP disappears from serum after birth is reduced in BALB/c/J mice. As a result, a true basal AFP level was not reached during the period studied by us. The reduced rate of AFP disappearance in BALB/c/J mice is not due to a reduced rate of AFP catabolism in these mice (Fig. 3), but appears to be due to an increased rate of AFP synthesis. There are several possible reasons for this increased AFP synthesis. Mice of the BALB/c/J strain could, for example, have reduced amounts of a factor that suppresses AFP production (10), or they could have a defect in the synthesis of a hormone that regulates AFP synthesis. Another possible reason for the increased synthesis of AFP is that there could be an increased turnover of liver cells in BALB/c/J mice. Since liver regeneration is associated with AFP production (5, 9), such an increased turnover rate would probably be correlated with an increased serum level of AFP. Examination of livers from BALB/c/J mice did not show any macroscopic or microscopic differ-

ence in liver structure between these mice and other mice. While this does not rule out an increased rate of cell division in BALB/c/J livers, it makes it unlikely that these mice suffer from a liver disease such as hepatitis, which causes liver regeneration and elevated serum AFP levels.

It is noteworthy that the initial rate with which AFP disappears from serum is rather similar in BALB/c/J mice and the other strains tested, as shown in Fig. 2. It is not until an age of 2–3 wk that the rate strongly decreases in BALB/c/J mice. One possible explanation for this finding could be that AFP is produced by two different cell populations, one major and one minor population. Normally, AFP production would be turned off in both populations shortly after birth, but in BALB/c/J mice only the major population would be efficiently turned off at this stage while AFP production in the minor population would continue and be turned off only slowly. In this connection it should be kept in mind that the AFP synthesized by BALB/c/J mice could be formed in some organ other than the liver, although this does not seem likely, since AFP synthesis takes place almost exclusively in the liver and the yolk sac of the fetus (11). Finally, it is of interest to note that increased levels of AFP have been observed in patients with cystic fibrosis (12). Like the property described in this paper, cystic fibrosis is inherited as an autosomal recessive trait, and it seems conceivable that the two traits are related.

Further work is now in progress to define the defect in BALB/c/J mice in more detail. It should, for example, be of interest to know whether it is the number of cells producing AFP or the amount of AFP produced per cell that is increased in BALB/c/J mice. Another point of interest is whether the *Raf* gene affects the susceptibility to liver carcinogenesis. In any case, the *Raf* gene could become a useful tool for studying the mechanisms that normally turn off AFP synthesis as well as the mechanisms that cause resumption of AFP synthesis in malignant hepatocytes.

Summary

To approach the genetic mechanism that turns off the synthesis of alpha-fetoprotein (AFP) after birth, we assumed that a change in this mechanism might affect the low basal level of AFP that can be detected in the adult organism. The concentration of AFP was therefore determined for serum from adult mice of 27 different inbred strains. With one exception, this basal level was between 34 and 173 ng/ml, which is about 10^5-fold less than the serum concentration at birth. In one strain, BALB/c/J, the AFP level was found to be considerably increased; it was about 10-fold higher than in other strains at 9–10 wk of age. Two other substrains of BALB/c mice showed normally low AFP levels. Kinetic studies show that the rate with which AFP disappears from serum after birth is reduced in BALB/c/J mice as compared to other strains. The increased AFP level of BALB/c/J mice appears to be due to an increased rate of synthesis of AFP, since the rate of catabolism of AFP was found to be normal in these mice. Genetic analysis was performed by crossing BALB/c/J mice with mice having an ordinary AFP level, followed by determination of AFP levels in mice of the F_1 and F_2 generations as well as in back-cross mice. The results clearly indicate that the increased AFP level in BALB/c/J mice is controlled by a single

recessive Mendelian gene, which has been named *Raf* (for regulation of alpha-fetoprotein). The *Raf* gene could be directly involved in the regulation of AFP synthesis, but it may also control AFP levels only indirectly, e.g., by regulating the synthesis of a hormone that controls AFP synthesis.

We thank Mrs. Lena Stensson for excellent technical assistance, Dr. Hans Nordgren for histological examination of mouse livers, and Professors Lars Kjellén and Rune Grubb for support and advice.

Received for publication 29 November 1976.

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